BLOOD CLOTTING AND ALLIED PROBLEMS

Transactions of the Fifth Conference
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Edited by

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Fifth Conference on Blood Clotting and Allied Problems

JOSIAH MACY, JR FOUNDATION CONFERENCE PROGRAM

As an introduction to these Transactions of the Fifth Conference on Blood Clotting and Allied Problems I should like to outline what it is that the Foundation hopes to accomplish by its Conference Program We are interested first of all in furthering knowledge about blood clotting and allied problems and to this end the participants were brought together to exchange ideas experiences data and methods In addition to this particular goal however there is a further and perhaps more fundamental aim which is shared by all our conference groups This is the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and a very urgent one the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another channels must be established for the most relevant dissemination of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science is a serious obstacle to scientific progress Particularly is it so in medicine that the limited view through the lens of one discipline is no longer enough For example today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation At the other extreme medicine is certainly a social science and through mental health must be concerned with economic and social questions The answer then is not further fragmentation into increasingly isolated specialties disciplines and departments but the integration of science and scientific knowledge for the enrichment of all branches This integration we feel can be encouraged by providing opportunities for a multiprofessional approach to given topics

Although the fertility of the multidiscipline approach is recognized adequate provision is not made for it by our universities scientific societies and journals And perhaps the presence of other hindering factors must be admitted Partly semantic in nature they

may also to some degree be psychological Admittedly it is often times difficult to accept data derived from methods with which one is unfamiliar By making free and informal discussion the central core of our meetings we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers

Thus our meetings are in contrast to the usual scientific gatherings They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice For this reason we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting Our hope is that the participants will come prepared not to defend a single point of view but to take advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories

We have now thirteen groups functioning under the Conference Program on the following topics adrenal cortex aging blood clotting cold injury connective tissues consciousness cybernetics infancy and childhood liver injury metabolic interrelations nerve impulse renal function and shock and circulatory homeostasis When a new conference is organized the Chairman in consultation with the Foundation selects fifteen scientists to be the nucleus of the group and *every effort is made to include representatives from* all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting but for the purpose of promoting full participation by all members and guests attendance at any meeting is limited to twenty five It is inevitable that in no topic can we possibly include more than a *small fraction* of the key investigators in the field and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include

The transactions of these meetings are recorded and published This is done because the Foundation wishes to make current thinking in a field available to all those working in it and because it believes that conveying to those in other fields who are concerned with science for example government officials administrators etc the essential nature of scientific research is also an important problem in communication Logic is a vital aspect of science but equally essential is the intuitive or creative aspect Research is as creative as the painting of a portrait or the composing of a symphony Al

though logic is of course necessary in order to rearrange to test and to validate research thrives on creativity which has its source in unconscious nonrational processes Unfortunately however in the finished products which are presented to the world through research reports this integral part of scientific endeavor is shriveled by the cold white light of logic By preserving the informality of our conferences in the published transactions we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays

FRANK FREMONT SMITH M D
Medical Director

CLINICAL EVALUATION OF THE NEWER ANTICOAGULANTS

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University of Minnesota

THE FIRST PART of this presentation is based on studies of the comparative effects of the three coumarin compounds dicumarol tromexan and 4 hydroxycoumarin anticoagulant No 63 (Figure 1) The action of bishydroxycoumarin U S P (dicumarol) is well

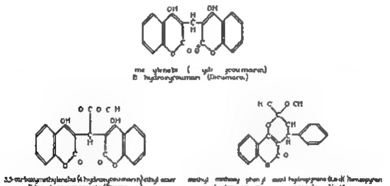

FIGURE 1 Structural formulas of bishydroxycoumarin ethyl biscoumacetate and 4 hydroxycoumarin anticoagulant No 63 Reprinted by permission from Barker N W Hanson H H and Mann F D Bishydroxycoumarin ethyl biscoumacetate and 4 hydroxycoumarin anticoagulant No 63 *J A M A* **148** *274 (1952)*

known to this group and will not be reviewed Tromexan (1 2) also called in the literature "pelantan B O E A "D E A and recently "ethyl biscoumacetate (N N R) was discussed two years ago at this Conference by Burke and Wright All reported studies of the effects of this compound on human beings are in agreement that it is about one fifth to one sixth as potent milligram for milligram as is dicumarol that it acts more rapidly than dicumarol and that its effect disappears more rapidly after administration is discontinued This is illustrated in Figure 2 In other respects the

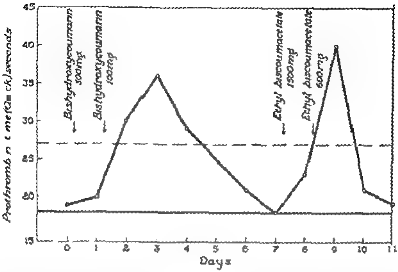

FIGURE 2 Comparative effects of equivalent doses of bishydroxycoumarin and ethyl biscoumacetate given to the same patient Reprinted by permission from Barker N W Hanson H H and Mann F D Bishydroxycoumarin ethyl bis coumacetate and 4 hydroxycoumarin anticoagulant No 63 *J A M A* 148 274 (1952)

action of tromexan is considered to be similar to that of dicumarol Side reactions and other untoward effects among human beings who have received therapeutic doses of tromexan appear to be rare and of little clinical consequence as is also the case with dicumarol The difference in speed of action and disappearance of action of these two drugs may be due to differences in solubility tromexan being the more soluble

The effect of fixed doses of tromexan like that of fixed doses of dicumarol varies considerably from patient to patient as is indicated in Figure 3 Some of the factors which may be responsible for these varying responses may be predicted in advance while others may not As is also true for dicumarol it is necessary to determine by trial and error the daily requirements of tromexan for the produc tion of "therapeutic" hypoprothrombinemia This means that on the basis of the response to one or more original fixed doses one must continue to adjust the dose and frequency of dosage according to the daily prothrombin level In this presentation the term hypo prothrombinemia" will be used to indicate the defect in coagulation of the blood indicated by prolongation of the one stage prothrombin time

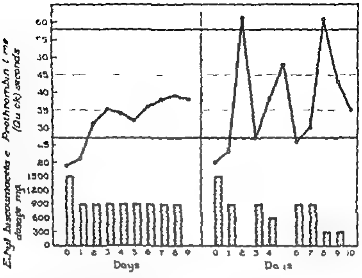

FIGURE 3 Effect of ethyl biscoumacetate on two patients showing variation in response of prothrombin time Response on left indicates a relatively resistant patient and response on right indicates a relatively sensitive patient with marked fluctuations of prothrombin time from day to day Reprinted by permission from Barker N W Hanson H H and Mann, F D Bishydroxycoumarin ethyl biscoumacetate and 4 hydroxycoumarin anticoagulant No 63 J A M A 148 274 (1952)

The third of the coumarin derivatives which I shall discuss is less well known It was developed in Dr Link s laboratory (3) by Ikawa and its effects on animals were studied by Scheel It was named "4 hydroxycoumarin anticoagulant No 63" by Link More recently it has also been called "cyclocumarol" and it is known by the trade names of "BL 5" and "cumopyran" Clinical studies on the effects of anticoagulant No 63 have been reported by Battle Capps Orth and Meyer (4) Rotter and Meyer (5) and recently by Hanson Mann and myself (6) It has been stated that in animals in which severe degrees of hypoprothrombinemia have developed after prolonged administration of large doses of anticoagulant No 63 mortality rates were lower and bleeding was less frequent than when similar levels of hypoprothrombinemia were induced and maintained by comparably potent doses of dicumarol Present clinical studies with anticoagulant No 63 indicate that its action is similar to that of dicumarol but that it is two to three times as potent milligram for milligram It is less soluble than dicumarol appears to act approximately as rapidly as dicumarol and its effect is maintained longer after administration is discontinued The re

sponse to certain fixed doses like the response to dicumarol and tromexan varies among different patients (Figure 4) Because of

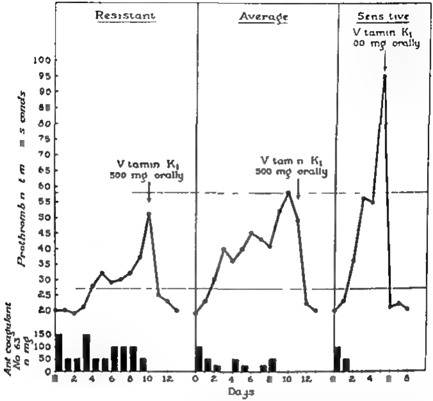

FIGURE 4 Varying responses of three different patients to anticoagulant No 63 the prothrombin time of each returned to normal rapidly after administration of vitamin K Reprinted by permission from Hanson H H Barker N W and Mann F D Clinical experiences with 4 hydroxycoumarin anticoagulant No 63 and the antagonistic effect of menadione and vitamin K *Circulation 4 814 (1951)*

its prolonged action satisfactory hypoprothrombinemia may be maintained with less frequent doses than is the case with dicumarol and tromexan After hypoprothrombinemia of the desired therapeutic range is attained additional doses are necessary as a rule only when the prothrombin time is decreasing (Figure 5)

In order to compare the effects of these three coumarin compounds I shall present data accumulated from studies of one hundred patients who received dicumarol one hundred patients who received anticoagulant No 63 and fifty patients who received tromexan Each patient was considered to have a specific indication

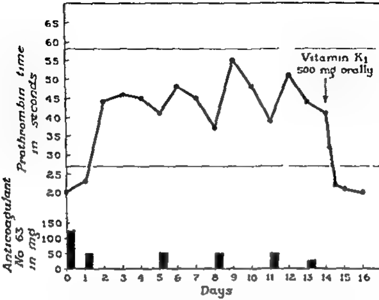

FIGURE 5 Relatively constant hypoprothrombinemia within the therapeutic range maintained for 14 days by infrequent doses of anticoagulant No 63 and then terminated rapidly after administration of vitamin K Reprinted by permission from Hanson H H Barker N W and Mann F D Clinical experiences with 4 hydroxy coumarin anticoagulant No 63 and the antagonistic effect of menadione and vitamin K *Circulation 4 844 (1951)*

for anticoagulant therapy The patients were unselected otherwise and in each of the three groups the number of patients with similar diseases was relatively proportionate Except for certain post operative patients the initial or priming dose was 300 mg of dicumarol 1 500 mg of tromexan and 150 mg of anticoagulant No 63 When the administration of the anticoagulant was begun within seventy two hours postoperatively the original or priming dose was two thirds of these amounts Subsequent doses of the three drugs for all patients on any day that they were given were as follows 50 to 150 mg of dicumarol 300 to 900 mg of tromexan and 25 to 75 mg of anticoagulant No 63 Whether or not these doses were given on each subsequent day during the period of treatment depended on the response of the patient The entire amount of each drug given on any one day was administered in a single dose Attempts were made to secure and maintain by these dosage schedules a hypoprothrombinemia within the therapeutic range" a range which has been arbitrarily designated at the Mayo

Clinic as indicated by prothrombin times between 27 and 58 seconds These figures correspond to the prothrombin times of normal plasma diluted to 30 per cent and 10 per cent of normal respectively * The normal prothrombin time in our laboratory for hospital patients is 18 to 20 seconds A saline extract of dried rabbit s brain is used as the thromboplastin

Table I

Initial Response

Anticoagulant Drug	Patients	Days needed to reach therapeutic range * per cent of patients in each group				
		1	2	3	4	Not reached in 5 or more
Dicumarol	100	19%	43%	16%	14%	8%
Tromexan	50	40%	44%	2%	4%	10%
Anticoagulant No 63	100	5%	80%	11%	4%	0%

* Days after administration was started until prothrombin time was first in therapeutic range as defined in text

A summary of the initial responses of the patients to the three drugs is given in Table I In this Table, it will be noted that a considerably larger percentage of patients receiving tromexan had prothrombin times within the therapeutic range at the end of the first twenty four hours At the end of forty eight hours the percentage of each group who had prothrombin times within the therapeutic range was essentially the same for both tromexan and anticoagulant No 63 and somewhat less in the group that received dicumarol No patients were entirely resistant to anticoagulant No 63 but a few were resistant to dicumarol (8 per cent) and tromexan (10 per cent) The usual doses of anticoagulant No 63 produced satisfactory hypoprothrombinemia in five patients who had been completely refractory to dicumarol during a week s trial Table I also indicates the variability of the initial response among different patients regardless of which drug was given The most consistent response was obtained in those who received anticoagulant No 63

* Editor s Note These dilutions refer to the plasma on which the prothrombin test was done not to the concentration of the plasma in the final clotting tube

Table II

**Persistence of Hypoprothrombinemia
After Cessation of Administration**

Anticoagulant drug	Patients	Days from last dose of drug to first normal prothrombin time	
		Range	Mean
Dicumarol	50	2 9	5
Tromexan	22	2 5	3
Anticoagulant No 63	22	5 14	8

Table II summarizes the means and ranges of duration of time between the last dose of the anticoagulants and the first normal prothrombin time in those patients who were not given antagonists and on whom prothrombin times were determined each day until it became normal The mean was shortest in those patients who received tromexan and longest in those who received anticoagulant No [illegible] *There was however considerable variation in this time* interval among the patients in each group and even among those who received tromexan this period was occasionally as long as five days The prothrombin time was usually only a few seconds above normal one to two days before it actually became normal hence the actual duration of *significant hypoprothrombinemia* was usually less than is stated in Table II

Our *experience with tromexan in the doses used showed* that in spite of attempts to individualize dosage rather wide fluctuations of the prothrombin time frequently occurred particularly during the first ten days of treatment (Figure 6) These fluctuations were noted especially in patients who appeared to be somewhat hyper sensitive to the drug In twenty four such patients the prothrombin time exceeded the therapeutic range twenty-one times and dropped below the therapeutic range eighteen times It was often impossible to predict on any given day whether or not another dose of the drug would cause the next day s prothrombin time to rise fall or remain approximately the same Similar fluctuations in the pro thrombin time have been noted in patients who received dicumarol but they are less marked In contrast fewer fluctuations from day to day were noted in patients receiving anticoagulant No 63

Clinic as indicated by prothrombin times between 27 and 58 seconds These figures correspond to the prothrombin times of normal plasma diluted to 30 per cent and 10 per cent of normal respectively * The normal prothrombin time in our laboratory for hospital patients is 18 to 20 seconds A saline extract of dried rabbit's brain is used as the thromboplastin

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serious consequence In one patient who received tromexan clinical evidence of thrombosis developed in the deep veins of the calf during treatment and at a time when the hypoprothrombinemia was within the therapeutic range (thirty seconds) No other evidence of thrombosis was noted in any of the three groups during treatment Gripe (7) recently reported serious bleeding in three of one hundred and twenty five patients who were being treated with tromexan The hemorrhages of two were nearly fatal and that of the third was fatal

Fremont Smith Did any of the hemorrhages occur when the prothrombin time was not particularly elevated?

Barker I am not certain about the cases reported by Gripe but in all of our cases the bleeding occurred when the prothrombin time was in the so called therapeutic range

Fremont Smith So that they were unexpected hemorrhages not predictable by prothrombin time?

Barker They were not entirely unexpected In our experience with dicumarol we have found that even with carefully controlled therapy and with the prothrombin time maintained within the therapeutic range instances of bleeding occur occasionally Most of this arises from potential bleeding lesions such as recent surgical wounds or ulcerative lesions in the gastrointestinal tract which have potentialities for bleeding even if no anticoagulant has been administered Perhaps the anticoagulant does not cause the bleeding but greatly exaggerates what might have been a very minor degree of bleeding from a particular lesion

Comparison of tromexan with dicumarol indicates that the more rapid return of the prothrombin time to normal when administration of tromexan is discontinued constitutes an increased safety factor On the other hand the more prolonged return of the prothrombin time to normal when administration of anticoagulant No 63 is discontinued adds an increased hazard to therapy with this anticoagulant This view is based on the fact that occasionally it is desirable to stop anticoagulant action as rapidly as possible for example when bleeding occurs or surgical operation becomes necessary However the differences in prolongation of effect of the three anticoagulants may be offset by the antagonistic effects of vitamin K_1 Several reports in the literature indicate that vitamin K_1 has a considerably more rapid and more certain antagonistic action to the effects of dicumarol (8 9 10) and anticoagulant No 63 (5 6) than do the water soluble synthetic menadione preparations Figures 7 and 8 indicate the comparative effectiveness of vitamin K_1

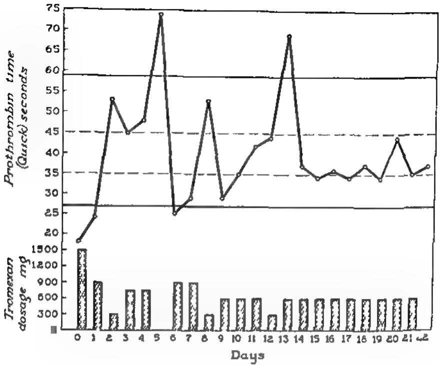

FIGURE ▮ . Fluctuations in prothrombin time produced by tromexan

Table III
Bleeding During Anticoagulant Therapy

Anticoagulant	Patients treated	Major bleeding	Minor bleeding
Dicumarol	100	1	3
Tromexan	50	1	2
Anticoagulant No 63	100	1	5

The incidence of major and minor bleeding among the three groups of patients is given in Table III Major bleeding occurred in only one case in each group Each of these patients had had a total hysterectomy and the bleeding was from vaginal wounds The prothrombin time of each rapidly fell to normal after vitamin K_1 was given and bleeding stopped before a serious amount of blood had been lost The minor bleeding was not considered to be of

and menadione sodium bisulfite in returning the prothrombin time to normal in the same patients after it has been elevated by dicumarol and anticoagulant No 63 respectively Vitamin K_1 may be given intravenously as a dilute emulsion without untoward effect but its action appears to be only slightly more rapid when given orally unless of course the patient is vomiting or has an external biliary fistula As an antagonist for the coumarin compounds the maximal effective dose of vitamin K_1 appears to be 500 mg In most instances however 100 to 250 mg appears to be just as effective It may be argued that the doses of 72 mg of menadione sodium bisulfite and 500 mg of vitamin K_1 (Figure 8) are not comparable but the doses of 141 mg of menadione and 250 mg of vitamin K_1 (Figure 7) are approximately comparable at least on a molar basis Other studies have shown that even 50 mg of vitamin K_1 is considerably more potent than 72 mg of menadione sodium bisulfite

Fremont Smith It seems to me that in Figures 7 and 8 there is very little evidence of any effect from the menadione sodium bisulfite

Barker I agree

Flynn Is this the same patient?

Barker Each Figure represents tests which were run consecutively on a single patient The same procedure was repeated in a number of patients both with dicumarol and anticoagulant No 63 the results were similar in all In some patients the doses of dicumarol were repeated a fourth time and a rise and fall of the prothrombin time occurred which was almost identical in degree and duration to that noted in the control period

Flynn In your experiment (Figure 7) is it possible that the initial administration of menadione sodium bisulfite acted as a "priming dose" causing the subsequent administration of vitamin K_1 to be more effective? Would you obtain the same results if the order was reversed? Quick and Collentine (11) also reported a greater antagonistic effect of vitamin K_1 but they too gave the menadione first

Barker We did not reverse the order chiefly because some patients may be somewhat refractory to coumarin compounds for as long as two weeks after being given vitamin K_1 particularly after doses as large as 500 mg We wished to have as nearly as possible the same response to the coumarin compounds each time Furthermore I think the possibility you mentioned is very unlikely since the immediate response to the same doses of dicumarol was the same

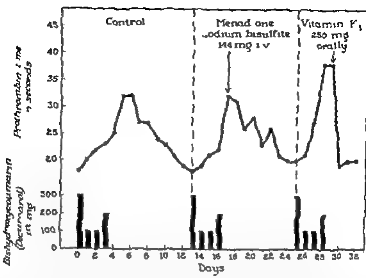

FIGURE 7 Comparative effects of menadione sodium bisulfite and vitamin K on the hypoprothrombinemia induced by bishydroxycoumarin in the same patient Reprinted by permission from Barker N W Hanson H H and Mann F D Bishydroxycoumarin ethyl biscoumacetate and 4 hydroxycoumarin anticoagulant No 63 *J A M A 148 274 (1952)*

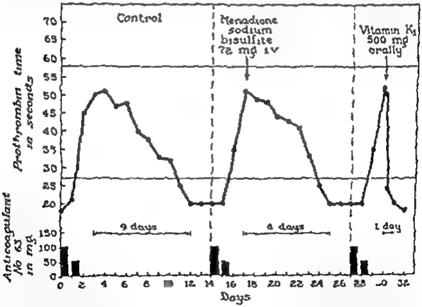

FIGURE 8 Comparison of effects of 72 mg of menadione sodium bisulfite given intravenously and 500 mg of vitamin K given orally on the hypoprothrombinemia induced by anticoagulant No 63 in the same subject Reprinted by permission from Hanson H H Barker N W and Mann F D Clinical experiences with 4 hydroxycoumarin anticoagulant No 63 and the antagonistic effect of menadione and vitamin K *Circulation 4 844 (1951)*

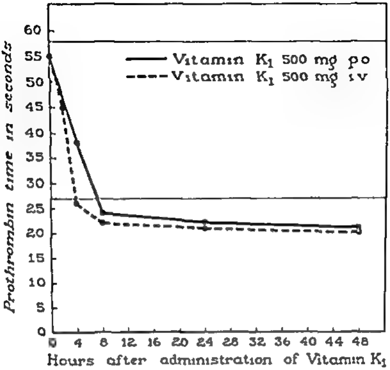

FIGURE 9 Rapid fall in the prothrombin time produced by vitamin K_1 either orally or intravenously

In spite of the poor effect of menadione sodium bisulfite noted in the studies indicated by Figures 7 and 8 it has been my experience and that of Overman Sorenson and Wright (12) that menadione sodium bisulfite is frequently effective in lowering the prothrombin time of hypersensitive patients who have excessive hypoprothrombinemia following the usual dose of anticoagulants It appears to be easy to restore the prothrombin activity to 20 to 30 per cent of normal with menadione but difficult to decrease the time which would ordinarily elapse before activity rises from 30 to 100 per cent of normal At present I think that the menadione preparations should be used if it is desirable to bring an excessively prolonged prothrombin time back into the therapeutic range but that vitamin K_1 should be used if it is desirable to bring the prothrombin time back to normal as rapidly as possible

Table IV shows the results of some studies on the effect of a single injection of 72 mg of menadione sodium bisulfite on exces

or even a little greater after the menadione sodium bisulfite than it had been just before the menadione sodium bisulfite

Alexander Does a patient who is refractory to No 63 after vitamin K_1 therapy respond readily to dicumarol?

Barker I have not done any experiments which would answer that question but I do not believe such a patient would respond any better to dicumarol than to anticoagulant No 63

Vitamin K_1 may be administered orally and it is effective by that route It may be administered intramuscularly and it may be administered intravenously In general the technique for intravenous use is to dissolve the amount of vitamin K_1 which is to be administered in approximately 10 ml of absolute alcohol and add to this 500 ml of a diluent either 1 per cent sodium chloride or 5 per cent glucose solution The resulting mixture is an emulsion which looks like dirty dishwater Because of its bad appearance one may be hesitant to inject it intravenously but it has been so given by gravity to a good many patients and to my knowledge there have been no untoward reactions Very finely divided emulsions in concentrated forms have been prepared and made available for experimental purposes but I think it is better to inject these very slowly or else to dilute them before injecting

Flynn Do supersonic waves clear vitamin K_1 emulsions?

Barker I don t know Dr Flynn I haven t tried that

Tocantins How rapidly does vitamin K_1 act?

Barker Figure 9 shows the comparative effects of vitamin K_1 when it was given intramuscularly and when it was given orally to a patient who had received anticoagulant No 63 At the end of four hours after intravenous administration and to a lesser degree after oral administration there is a fall in the prothrombin time but not down to the lower limit of the therapeutic range At the end of eight hours after intravenous injection the prothrombin time has usually fallen below the therapeutic range After oral administration the prothrombin time has dropped further but usually not below the therapeutic range at the end of eight hours At the end of twenty four hours the prothrombin time is usually down to normal after either oral or intravenous administration of 500 mg except in a few patients perhaps 10 to 15 per cent even in these it is rarely more than two seconds above normal

Tocantins Does it ever become shorter than normal?

Barker Not when measured by our standard technique for the one stage test We have not tried your technique of using concentrated reagents

effect than either dicumarol or tromexan and when it is used it is easier to maintain a relatively constant degree of therapeutic hypoprothrombinemia Although adequate statistical information is still lacking it appears probable that if a similar degree of hypoprothrombinemia is induced and maintained use of each of the three drugs has approximately the same value in preventing thrombosis and is attended by approximately the same risk of bleeding

A few comments may be in order on the status of the heparin substitutes Paritol C was discussed at two of our previous Conferences (1949 and 1950) by Irving Wright Since that time Bartholomew and I have found that protamine sulfate would rapidly neutralize the anticoagulant effect of paritol C Approximately a year ago a report was sent out from the manufacturer summarizing studies by 27 clinical investigators who had given 925 doses of paritol C to 247 patients In this group there had been seven severe hypotensive reactions one moderate hypotensive reaction one systemic reaction without hypotension two internal hemorrhages three gastrointestinal reactions and five instances of paresthesia One patient died after complete heart block developed and one after a severe convulsive seizure In spite of possibly extenuating circumstances such as injections given too rapidly the fact that these reactions did occur has led most investigators to abandon the use of paritol C and it is likely that concern regarding the possibility of serious reactions will preclude any extensive further investigation of this anticoagulant

Another substitute for heparin has been the subject of two recent reports (12 13) This preparation is stated to be the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside and is called "treburon" by the manufacturer

Link That is an old product which was made in our laboratory back in 1936 We did not pursue it further at that time because we were too busy

Barker Mangieri Engelberg and Randall (13) have reported on its action in animals Hirschboeck Madison Giliberti and Pisciotta (14) have reported administration of this preparation to one hundred and forty two patients the majority of whom were hospitalized for cardiovascular disease without any toxic reactions They found that the drug has a heparin like action when administered intravenously and has about one third the potency of sodium heparin Thus 150 mg produced an effect on the coagulation time similar to that of 50 mg of sodium heparin They also found an effective anticoagulant action which lasted twelve hours or longer

Table IV

Effect of 72 mg of Menadione Sodium Bisulfite on the Hypoprothrombinemia Induced by Tromexan

Patient	Prothrombin time in seconds	
	Before injection	16-20 hours after intravenous injection
1	145	27
	125	25
2	121	25
3	93	23
4	80	25
5	57	28
6	47	20

sive hypoprothrombinemia produced by tromexan It will be noted that sixteen to twenty hours after the injection the prothrombin times were markedly reduced but in only one instance had the prothrombin time returned to normal It may be questioned whether this decrease in prothrombin time is greater than would have occurred by simply discontinuing administration of the tromexan without giving the menadione The effect of vitamin K_1 in returning the prothrombin time to normal was approximately the same in a limited number of patients studied who had received tromexan as that noted in patients who had received dicumarol or anticoagulant No 63 Thus if vitamin K_1 is available and it becomes necessary to stop the effect of an anticoagulant as rapidly as possible it is probable that vitamin K_1 will do it with approximately equal rapidity whether the anticoagulant has been tromexan dicumarol or anticoagulant No 63

In summary I believe that from the clinical standpoint tromexan is superior to dicumarol in that usually it produces hypoprothrombinemia more rapidly but it is inferior to dicumarol in that greater difficulty is experienced in maintaining the hypoprothrombinemia within the therapeutic range during the first one or two weeks of treatment Anticoagulant No 63 has a somewhat more constant

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6 HANSON H H BARKER N W and MANN F D Clinical experiences with 4 hydroxycoumarin anticoagulant No 63 and the antagonistic effect of menadione and vitamin K_1 *Circulation 4 841 (1951)*
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12 SORENSON C W and WRIGHT I S Synthetic anticoagulant polysulfuric acid ester of polyanhydromannuronic acid (Paritol) experience with its use in man *Circulation* 2 *658 (1950)*
13 MANGIERI C N ENGELBERG R and RANDALL L O The heparin like activity of a new anticoagulant treburon *J Pharmacol & Exper Therap* 102 *156 (1951)*
14 HIRSCHBOECK J S *et al* A clinical study of the anticoagulant properties of treburon *Wisconsin M J* 50 *863 (1951)*

after intramuscular administration of doses averaging 625 mg and a similarly effective action during slow, continuous intravenous administration of a 5 per cent solution of glucose containing 600 to 800 mg of treburon per liter

Scholz and I have had some limited experience with treburon which has tended to confirm the work of Hirschboeck and his associates When 150 mg of treburon was given intravenously the effect was approximately as intense and as prolonged as that of 50 mg of heparin Somewhat more intense and longer effects were achieved by doses of 200 mg In three cases we found that the anticoagulant effect of treburon injected intravenously was rapidly neutralized by the intravenous injection of half the amount in milligrams of protamine sulfate One rather nervous patient had slight nausea after injection of treburon but no other untoward reaction of any type was encountered in our cases In a recent personal communication to me Hirschboeck stated that treburon has been given to eighty additional patients without any untoward reactions except that one patient after three and a half weeks of intramuscular injections of treburon gradually lost about half of the hair of her scalp It was uncertain that this was caused by treburon since other patients who had been treated for longer periods had not given evidence of alopecia Thus the studies to date suggest that treburon may be an effective and apparently safe substitute for heparin Its only advantage over heparin however would seem to be the claim of the manufacturer that if and when it is placed on the market it will be less costly and that more sustained action may be obtained with intramuscular injections

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A COMPARATIVE EVALUATION OF TROMEXAN AND DICUMAROL

IRVING S WRIGHT
Department of Medicine
Cornell University Medical College

SOME OF THE data collected by the Committee on Anticoagulants of the American Heart Association (1) correlate very well with many of the points mentioned by Dr Barker In one of the studies done by this group an endeavor was made to evaluate the relative effects of dicumarol and tromexan in man This was carried out in seven hospitals and due credit should be given to the responsible investigators and the staffs of those hospitals for the work which made the compilation of the material herein presented possible* The material will be published in detail elsewhere but in summary the findings were as follows

A total of 514 patients with actual or threatened thromboembolic conditions were treated with one or both of these anticoagulants A total experience of 6 642 days of tromexan therapy and 5 006 days of dicumarol therapy (without supplementation by other anticoagulants) was reviewed and analyzed The responsible investigators from the seven hospitals cooperating in the project reported each individual case on a detailed master form to the Central Laboratory Analysis of findings from these cases has resulted in the following conclusions as to the relative advantages of the two anticoagulants

1) Previous reports that a more rapid initial prolongation of prothrombin time can usually be achieved with tromexan than with dicumarol have been confirmed (Figure 10) This characteristic makes it possible *to protect the patient more rapidly* with tromexan than with dicumarol in the initial stages of anticoagulant therapy

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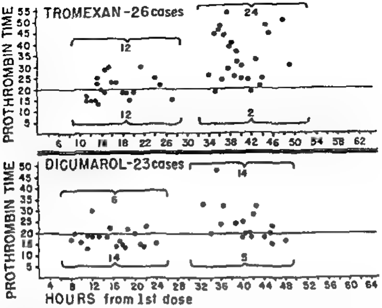

FIGURE 10 Scattergram showing the relative rates of prolongation of prothrombin time after 1500 to 1800 mg of tromexan plus 900 mg the second day and 300 mg of dicumarol, plus 200 mg the second day Compiled by Dr Louis Scarrone Reprinted by permission, from Wright I S The George Brown Memorial Lecture The pathogenesis and treatment of thrombosis *Circulation 5 178 (1952)*

when the risk of thromboembolic complications is usually especially high as well as after a lapse in therapy when a rapid return to therapeutic levels is indicated

2) *Previous reports that prothrombin times usually return more* rapidly to normal after the cessation of tromexan than after the cessation of dicumarol have been confirmed in a variety of circumstances This characteristic of tromexan is of advantage if it becomes advisable to terminate therapy as when excessively prolonged prothrombin times with or without bleeding develop or when emergency surgery becomes necessary (Figure 11)

3) The ability of the two anticoagulants to protect the patient from thromboembolic complications appears about equal as demonstrated by the close similarity in the thromboembolic complication rates for days of therapy under each of the two anticoagulants (Figures 12 and 13)

4) With tromexan more of the thromboembolic complications occurred when prothrombin *times were below the optimal therapeutic* range than was the case with dicumarol This suggests that

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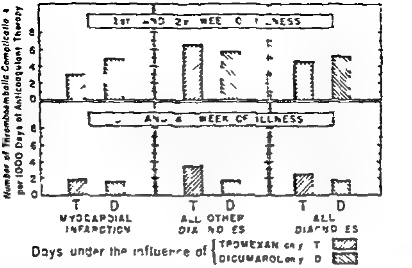


FIGURE 13 Number of thromboembolic complications of all types occurring on days when patients were under the influence of tromexan or dicumarol only per 1000 days of corresponding type of anticoagulant therapy by diagnosis and week of illness (cases with unknown date of onset and therapy after the 4th week omitted)

control of the lower limits of the therapeutic range presents more of a problem with tromexan than with dicumarol

5) Mild toxic reactions (nausea diarrhea or rashes) were *infrequent* being reported in 12 (4.2 per cent) of the patients treated with tromexan and in 1 (0.4 per cent) of the patients treated with dicumarol These reactions did not constitute a significant disadvantage in the use of either drug

6) A review of the laboratory data for all patients failed to reveal that either tromexan or dicumarol produced significant evidence of toxicity in the doses commonly used for therapy

7) Fourteen autopsies were studied in detail These did not reveal any morphological evidence for differences in the toxicity of the two anticoagulants or their effectiveness in preventing thromboembolic complications

8) A higher proportion of total prothrombin times were 50 seconds or more (i e a prothrombin level of 7 per cent or less) during tromexan therapy than during dicumarol therapy These were mostly encountered in patients on a single daily dose (Figure 14)

9) This tendency to excessive response was reduced markedly in those patients who received tromexan in divided daily doses

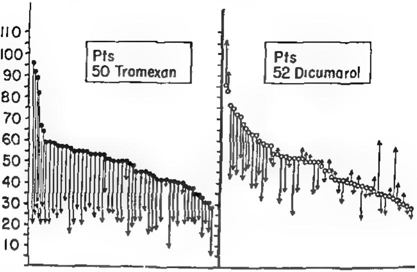


FIGURE 11 Extent and direction of change in prothrombin time within 36 hours after last dose of tromexan and dicumarol. No vitamin K or transfusions were given. In every instance following the termination of tromexan the trend was downward [illegible] safe levels. Thirty six hours after the termination of dicumarol 19 out of [illegible] patients showed prothrombin times which were still higher than the previous test. Compiled by Dr Louis Scarrone. Reprinted by permission from Wright I S The George Brown Memorial Lecture Pathogenesis and treatment of thrombosis *Circulation 5 178 (1952)*

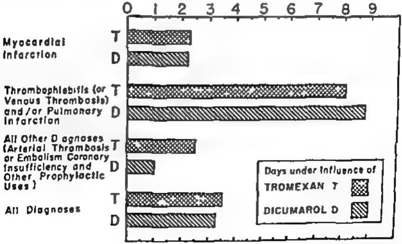


FIGURE 12 Number of thromboembolic complications of all types occurring on days when patients were under the influence of tromexan or dicumarol only per 1000 days of corresponding type of anticoagulant therapy by diagnosis (corrected rates)

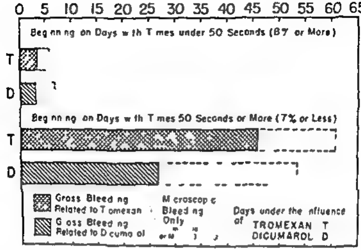


FIGURE 15 Number of gross and microscopic bleeding episodes due to or aggravated by anticoagulant therapy such episodes beginning on days when patients were under the influence of tromexan or dicumarol and showed prothrombin times under 50 seconds or 50 seconds or more Figures calculated per 1000 days of anticoagulant therapy characterized by times at similar levels

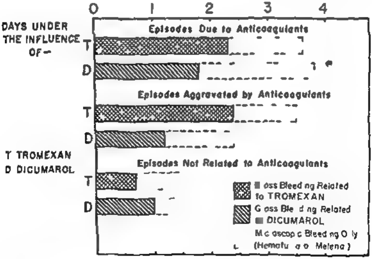


FIGURE 16 Number of gross and microscopic bleeding episodes due to aggravated by or unrelated to anticoagulant therapy episodes beginning on days when patients were under the influence of tromexan or dicumarol Figures calculated per 1000 days of corresponding type of anticoagulant therapy

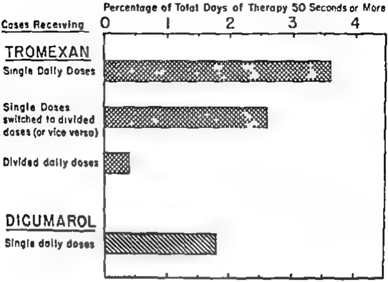


FIGURE 14 Percentage of total days spent under the influence of tromexan and dicumarol only and having prothrombin times of 50 seconds or more (a prothrombin level of 7 per cent or less) among patients receiving tromexan in single and/or divided daily doses and among patients receiving dicumarol in single daily doses

10) The total incidence of gross hemorrhage related to anticoagulants was slightly higher during tromexan therapy than during dicumarol therapy This difference was found primarily at times of 50 seconds or over and during the early stages of anticoagulant therapy (Figures 15 16 17 18)

11) When observations were limited to days when prothrombin times were under 50 seconds (i e a prothrombin level of above 7 per cent) the incidence of hemorrhagic episodes due to or aggravated by the two anticoagulants appeared approximately similar indicating that the observed differences in total hemorrhage rates (see *9*) were related to the more frequent upward fluctuations following tromexan in single daily doses and not to some inherent characteristic of this anticoagulant

12) These combined observations (*8* to *11*) suggest that when given in single daily doses tromexan is slightly more difficult to control than dicumarol Experience with the daily total divided into two or three doses indicates that the prothrombin time is more satisfactorily controlled with this regimen than with single doses (Figures 19 and 20)

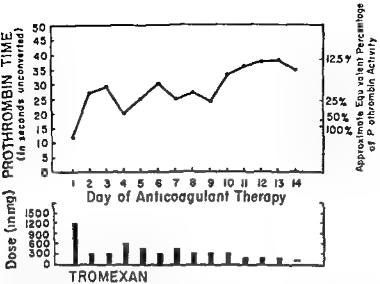


FIGURE 19 Case illustrating good control of prothrombin times after rapid prolongation following a small initial dose of tromexan given for acute thrombophlebitis (Note slight fluctuations in daily prothrombin times Drug was administered in a single dose)

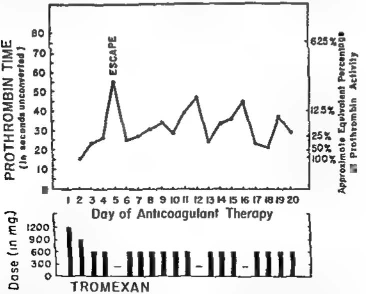


FIGURE 20 Case demonstrating sudden changes in prothrombin times with single doses in a patient receiving tromexan for acute thrombophlebitis (Cessation of drug for one day resulted in prompt fall in the next prothrombin time Note lack of response to small [1200 mg] initial dose)

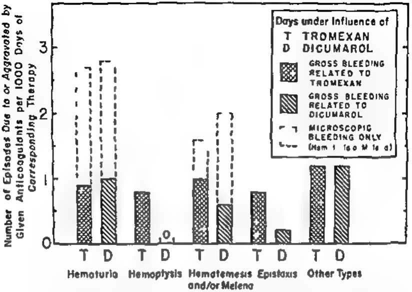


FIGURE 17 Number of bleeding episodes of specific types beginning on days when patients were under the influence of tromexan or dicumarol only and considered due to or aggravated by these anticoagulants Figures calculated per 1000 days of anticoagulant therapy

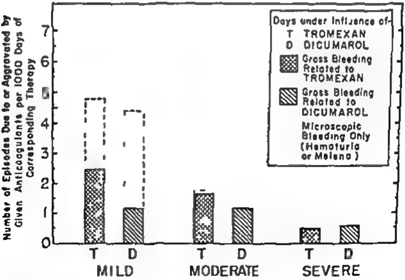
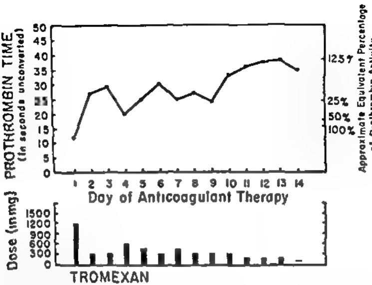


FIGURE 18 Number of mild moderate and severe gross and microscopic bleeding episodes due to or aggravated by anticoagulant therapy beginning on days when patients were under the influence of tromexan or dicumarol Figures calculated per 1000 days of corresponding anticoagulant therapy



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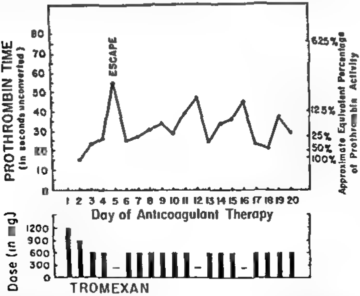

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13) In this series of patients two deaths occurred in which tromexan may have played a role In one of these poor control of prothrombin times was a factor Two deaths occurred in patients who were on a combination of paritol and dicumarol therapy The clotting times were prolonged in both and the prothrombin times were 54 and 22 seconds respectively It is difficult to assay the part played in these deaths by each of the two anticoagulants involved

14) As with all coumarin derivatives and phenylindandione the prothrombin times of patients receiving tromexan should be watched with especial care until the response pattern is fully evident Thereafter the use of anticoagulants requires meticulous supervision by the attending physician

15) The foregoing conclusions relate to the total experience with tromexan and dicumarol when employed in a variety of thromboembolic conditions In addition some comparisons are possible with the findings of a previous study on the use of dicumarol in myocardial infarction Some comparisons require however the omission of patients with diagnoses other than myocardial infarction These omissions reduce the sample to 262 patients of whom 139 received tromexan only 106 dicumarol only and 17 both anticoagulants The following conclusions pertain only to this component of the total sample

a) Of the myocardial infarction patients receiving dicumarol and no tromexan 14 2 per cent died within six weeks of the date of onset as compared with 10 8 per cent of the patients receiving tromexan and no dicumarol However since two of the dicumarol deaths occurred in association with the combined use of paritol and dicumarol and since slightly more of the tromexan cases were mild at onset and for other reasons these figures cannot be attributed to differences between the two anticoagulants Both death rates are slightly below the 16 0 per cent record of deaths within six weeks in the treated group in the previous dicumarol study and they are very substantially below the 23 4 per cent of deaths occurring in the control group in that study

b) The thromboembolic complication rates for the myocardial infarction patients were similarly favorable when compared with the results of the previous dicumarol study During tromexan therapy in the present study thromboembolic complications averaged 2 3 per thousand days of therapy and during dicumarol therapy the average was 2 2 per thousand days Both of these daily rates are about *one* third lower than the corresponding rate of 3 1 complications per thousand days during the period of dicu

marol therapy in the previous study Furthermore they are significantly below the 12 7 rate per thousand days for the control group in the dicumarol study

c) Thus the power of protection of the two anticoagulants in myocardial infarction does not vary greatly When judged by comparison with the control group of the previous study both produced significant reductions in death rates and in the incidence of thromboembolic complications The experience of this study with an additional 262 patients with myocardial infarction therefore constitutes further confirmation of the major conclusions of the original study

To discuss paritol briefly at a previous Conference we reported a shock like reaction to this drug At that time Dr Link said it was his belief that paritol belonged to a group of compounds from which such reactions could be expected His statement has been borne out by further experience with paritol since there have been additional reports of shock like reactions from other investigators for example Dr Barker s comment earlier We have seen patients whose hands and feet became edematous for a few hours after paritol We have no explanation for this very curious phenomenon A number of patients who have received paritol have developed alopecia about six weeks afterwards That is particularly interesting in light of the experience with treburon It has also been reported as occurring following the use of compounds similar to paritol This alopecia has been fairly complete in a number of patients although hair did return in all of them after a few weeks It is our feeling that the clinical use of paritol should be deferred until further studies can be carried out with reference to all possible toxic effects

This may be a good time to throw open for discussion the anticoagulants reviewed by Dr Barker and myself I am sure Dr Seegers has some comments

Seegers I should like to ask about the availability of vitamin K_1 and vitamin K_1 oxide Dr Barker where may one get these compounds and what are the prospects for the future?

Barker We have obtained our vitamin K_1 from Merck and Company Unfortunately they have made it commercially available only in bulk form Its cost is a little more than that of comparable amounts of the water soluble menadione compounds For oral use doses of the bulk preparation can be put into ordinary gelatin capsules If it is protected from light it seems to be stable and to retain its potency We have also obtained some concentrated emul

13) In this series of patients two deaths occurred in which tromexan may have played a role In one of these poor control of prothrombin times was a factor Two deaths occurred in patients who were on a combination of paritol and dicumarol therapy The clotting times were prolonged in both and the prothrombin times were 51 and 22 seconds respectively It is difficult to assay the part played in these deaths by each of the two anticoagulants involved

14) As with all coumarin derivatives and phenylindandione the prothrombin times of patients receiving tromexan should be watched with especial care until the response pattern is fully evident Thereafter, the use of anticoagulants requires meticulous supervision by the attending physician

15) The foregoing conclusions relate to the total experience with tromexan and dicumarol when employed in a variety of thromboembolic conditions In addition some comparisons are possible with the findings of a previous study on the use of dicumarol in myocardial infarction Some comparisons require however the omission of patients with diagnoses other than myocardial infarction These omissions reduce the sample to 262 patients of whom 139 received tromexan only 106 dicumarol only and 17 both anticoagulants The following conclusions pertain only to this component of the total sample

a) Of the myocardial infarction patients receiving dicumarol and no tromexan 14 2 per cent died within six weeks of the date of onset as compared with 10 8 per cent of the patients receiving tromexan and no dicumarol However since two of the dicumarol deaths occurred in association with the combined use of paritol and dicumarol and since slightly more of the tromexan cases were mild at onset and for other reasons these figures cannot be attributed to differences between the two anticoagulants Both death rates are slightly below the 16 0 per cent record of deaths within six weeks in the treated group in the previous dicumarol study and they are very substantially below the 23 4 per cent of deaths occurring in the control group in that study

b) The thromboembolic complication rates for the myocardial infarction patients were similarly favorable when compared with the results of the previous dicumarol study During tromexan therapy in the present study thromboembolic complications averaged 2 3 per thousand days of therapy and during dicumarol therapy the average was 2 2 per thousand days Both of these day rates are about one third lower than the corresponding rate of 3 1 complications per thousand days during the period of dicu

And frequently one is not interested in getting the prothrombin time back up in that particular patient at least for some time The main problem is to handle a critical situation immediately I think that is the real indication for vitamin K_1

Wright How would you compare the action of vitamin K_1 to whole fresh blood transfusions the effect of which would be over much more quickly?

Barker We only use whole fresh blood transfusions to replace blood not to replace prothrombin I think that the effectiveness of vitamin K_1 is far greater than that of whole blood in lowering the prothrombin time If the patient has lost enough blood so that his condition is serious then we give blood If the patient has not lost enough blood to cause symptoms or affect his hemoglobin concentration we only give vitamin K_1 watch him carefully and wait to see if he is going to need the blood

Brambel We likewise made an extensive study of the effectiveness of vitamin K_1 counteraction of oral anticoagulants as compared with whole blood Our findings are in complete agreement with Dr Barker's For example we had a patient with a prothrombin time of 60 seconds who after receiving 1 000 ml of whole blood had a prothrombin time of 90 seconds on the following morning (Figure 21)

Allen How long was that in hours?

Brambel That was about fifteen or sixteen hours

Wright Did you make any tests to see whether the prothrombin time had dropped in four or six hours after the transfusion?

Brambel No not in this particular one I want to point out that overnight the prothrombin time had increased despite the administration of two pints of blood

Allen Was the prothrombin time immediately determined at the end of the transfusion?

Brambel No

Allen That is a common mistake made in the biology of prothrombin activity There is a mathematical addition after rapid transfusion but the transfusion activity rapidly disappears in six to twelve hours

Wright Mathematical addition in terms of what?

Allen In terms of blood volume Such a response is obtained in dogs given dicumarol or rendered K deficient (Figure 22)

Alexander A few years ago we published observations on the disappearance rate of prothrombin in congenital hypothrombinemia after transfusions with fresh blood with observations made immedi

sion of vitamin K_1 in ampules from Merck and Company, but I do not believe that this has been made available commercially Some vitamin K_1 oxide in capsules and in emulsion has come from the Abbott Laboratories and a small amount of vitamin K_1 oxide in capsules from the Rowell Laboratories of Baudette Minnesota

Seegers I have one more question It was not clear to me whether or not vitamin K_1 oxide may also be given orally and whether the same results can be expected as with vitamin K_1

Barker So far we have found that the effect of vitamin K_1 oxide when given either orally or parenterally in the same amounts is essentially the same as the effect of vitamin K_1

Wright The question of vitamin K_1 producing a refractory period to the anticoagulant drugs is important Indeed the question can be brought into sharper focus by considering a patient with a thrombosis who begins to bleed Obviously the prothrombin time of this patient must be lowered preferably just enough to take the patient out of the hemorrhagic level but not low enough to permit further propagation of the thrombus In such a case vitamin K_1 will correct the hypoprothrombinemia but if too much vitamin K_1 is given then the patient may be refractory for a time to the effect of the anticoagulant drug permitting the thrombus to propagate This is really one of the key problems in relation to the use of compound No 63 since it responds less readily to the water soluble vitamin K preparations In the event of excessive hypoprothrombinemia one is almost forced to use vitamin K_1 or vitamin K_1 oxide in which case there may be a prolonged effect toward a normal prothrombin time sometimes with subsequent disastrous thromboembolic phenomena

Barker Refractoriness to coumarin compounds after vitamin K_1 is dependent somewhat on the amount of vitamin K_1 which has been given At first we used what had been stated in the literature to be the maximal therapeutic dose 500 mg After 500 mg most patients are somewhat refractory to coumarin compounds However we have rarely noted refractoriness to dicumarol or anticoagulant No 63 after doses of 250 mg of vitamin K_1 or less and 250 mg seems to be just as rapidly and completely effective in neutralizing the hypoprothrombinemia in almost all patients as is 500 mg I certainly agree that vitamin K_1 even in small doses is too potent an antagonist to use if the purpose is only to bring an excessively high prothrombin time back into the therapeutic range but situations arise occasionally where one wants to get the prothrombin time down to normal just as rapidly as possible

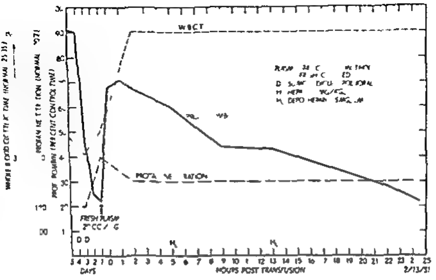

Figure 22. Effect of plasma on dicumarolized heparinized dog Dog 5__ weight 9 7 kg WBCT = whole blood clotting time

Brambel To return to the patient (Figure 21) whose prothrombin time increased after the transfusion the next morning 100 mg of K_1 emulsion was given intravenously and in a matter of six hours the prothrombin time was 22 seconds From our experience we know that vitamin K_1 in emulsified form will cancel the effects of the anticoagulants phenylindandione tromexan compound 63 and dicumarol However great confusion exists as to what is meant when we speak of vitamin K_1 In a review of the literature it is often found that when clinicians refer to vitamin K_1 they mean some derivative of menadione Menadione derivatives are not vitamin K_1 and they are certainly nowhere near as potent In our experience with their use in counteracting anticoagulant defects regardless of the dosage the prothrombin time values never go down to normal Eighteen or nineteeen seconds was the best we could obtain no matter how much or which derivative was used On the other hand vitamin K_1 will reduce prothrombin time to normal but will make the patient refractory to anticoagulants

Thus it seems to me two aspects of this problem have to be considered effectiveness and over correction When the problem is one of severe hemorrhage due to hypothrombinemia transfusions in a number of cases are of no avail However a preparation such as

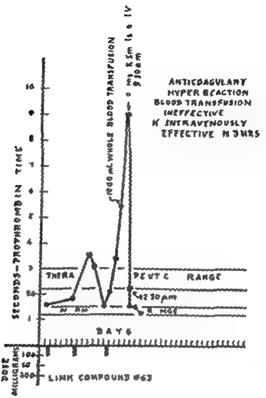

FIGURE 21

ately following transfusion and at periodic intervals thereafter (2) The rate at which the prothrombin activity disappeared from the patient s plasma was very rapid within twelve hours it was virtually absent

Wright Frequently however that drop seems to be sufficient to bring about a cessation of bleeding and allow bleeding points to seal over

Alexander I concur entirely

Wright In the case of a person who has a rapidly advancing process of thrombosis there is an advantage in doing something immediately but without holding the prothrombin time at a normal level for a long period

Alexander By a combination of the use of fresh blood and simultaneous administration of K_1 I think one has a most effective way of managing hemorrhage

min K_1 the bleeding has stopped In the three patients mentioned in Table III who had major bleeding we did not use transfusions All the bleeding stopped after the administration of K_1 in six hours or less I might add one other point The cost of K_1 is certainly far less than the cost of blood transfusions

Wright But greater than water soluble K

Barker Yes but only a little greater

Seegers My calculation about the number of prothrombin units that a human has would be in the neighborhood of a million One could get about a million units of purified bovine prothrombin into ■ ml of solution without any difficulty and thus restore the prothrombin to the desired level I am wondering whether there is any possibility of using a solution of that kind I know very well that the question of sensitivity would come up but patients would probably not be sensitive the first time it is given

Brambel The only trouble with that Dr Seegers is the length of time it would last In our experience with some of our human fractions we brought the prothrombin time down all right but in a matter of several hours it went up again

Wright If it is a matter of several hours there is no reason why patients cannot receive successive doses if they are not sensitized to it so that is not really an objection The key question is did it stop the bleeding?

Brambel No it didn t

Wright That is very important

Lewis We did try to prepare human prothrombin for a patient with idiopathic hypoprothrombinemia Most of the activity was lost during preparation and sterilization

Alexander How did you sterilize?

Lewis Both Seitz and Berkefeld filtration removed a good deal of activity

Seegers There should be no trouble in filtering prothrombin It can be put through a fritted filter without difficulty

Lewis Something happened to the human prothrombin Perhaps it was loss of accessory factors such as accelerin or convertin rather than prothrombin

Seegers You probably did not have it sufficiently purified

Overman I should like to ask Dr Barker if he has used any other thromboplastin preparation to determine whether or not he has observed this wide variation shown in Figure 6 as he does with his routine thromboplastin

Barker I believe we did in a very few cases but not consistently

vitamin K_1 given intravenously will produce effective changes within twelve hours But, in the absence of hemorrhage might not the menadione derivatives be effectively used to restore the clotting mechanism to nearly normal levels or within the nearly therapeutic range?

Allen I should like to take exception to your statement concerning the inadequacy of transfusions It has been our experience that transfusion is the only thing that will give an instantaneous boost of prothrombin activity If a patient who has been on dicumarol or any of the allied compounds for two weeks or longer develops hemorrhagic trouble it may be necessary to give blood or plasma every six hours

Brambel How long would it take to stop a severe gross hematuria with blood transfusions alone?

Allen If there is blood in the urine contained in the bladder the next urine voided will not give an indication of improvement unless the patient is catheterized immediately at the end of the transfusion There will not be bleeding for the next four to six hours after the transfusion if enough blood or plasma has been given or at least we have not had it But it will return if more transfusions are not given certainly within twelve to twenty four hours there will be a recurrence

Wright In some patients

Allen What we have been using is a combination of transfusion and vitamin K_1 because we have never felt that we could get out of serious trouble with K_1 alone when instantaneous correction of a hemorrhagic problem was required

Barker Our experience has been different which is one of the reasons why we have done so much work with vitamin K_1 In our patients who were bleeding we were unable to stop the bleeding with transfusions They kept right on bleeding We use vitamin K_1 rather than transfusions to stimulate natural replacement of prothrombin and accelerators

Allen Have you studied the prothrombin activity at fifteen minutes thirty minutes or an hour after transfusion?

Barker No but we have found the prothrombin time unchanged two or three hours after transfusion and the patient has continued to bleed during this time

Allen Even granting your premise would you not have to say that you do not know whether it is the elevation of prothrombin by K_1 or the transfusion that has stopped the bleeding?

Barker The practical point is that when we have given the vita

view it is not quite as much of a hazard as was feared originally

Barker I doubt that one can make a comparison between the anticoagulant action of heparin and the anticoagulant action of dicumarol as they are quite different

Allen It should be pointed out however that in spite of the fact that the prothrombin time returns to normal, it is amazing how long the clotting time of whole blood will stay out After four five or six days of dicumarol the clotting time of whole blood may remain prolonged for several weeks to two months after the drug has been discontinued

Wright How are the clotting times done?

Allen By a Lee White modification

Wright How many tubes do you use?

Allen Five tubes are used Often we obtain clotting times of 60 to 80 minutes while the normal varies from 25 to 40 minutes

Wright Following dicumarol?

Allen Yes after dicumarol is stopped

Owren When dicumarol was introduced in Norway it happened that a private practitioner started to use it without control and thus there were referred to our department a few patients with dicumarol intoxication Figure 23 illustrates the effect of transfusions in a case of severe intoxication

The method used for controlling the coagulability of the blood gives a quantitative expression of the combined effect of the decreased prothrombin and proconvertin* (the P & P method†) With transfusion of whole blood we found an increase in the P & P value of 6 7 or 8 per cent corresponding to the amount of transfused plasma but the effect had completely disappeared by the next day By doing controls we found that the effect of a transfusion lasted only about twelve hours The clinical symptoms especially hematuria lasted for several days after the transfusion treatment was started Daily transfusions of half a liter of blood therefore seemed not to stop the hematuria which persisted until the prothrombin synthesis in the liver had started and a concentration of about 40 per cent was reached Vitamin K as far as we could see had no effect in this case

Barker What kind of vitamin K?

Owren It was the water soluble 2 methyl 1 4 naphthoquinone

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* Editor's Note Owren's terminology for the precursor of a factor involved in the conversion of prothrombin to thrombin See appendix for the synonymy of this term as well as of the others used by Owren

† Editor's Note A method devised by Owren for measuring prothrombin and proconvertin

Overman It has been our experience that if we use a rabbit brain thromboplastin (Difco) or a rabbit lung thromboplastin as we prepare it in our laboratory we do not obtain this wide variation or daily fluctuation which you have found with tromexan One obtains a curve more like that observed when dicumarol or compound 63 are used The commercial lung preparations which we have tried give this wide fluctuation similar to the curve that you presented

We have observed this difference quite frequently and I wondered whether this has been the experience of other laboratories For example a prothrombin time of 30 to 40 seconds with rabbit brain thromboplastin (Difco) or with the rabbit lung thromboplastin which we prepare will routinely give us a prothrombin time of 60 to 80 seconds with some of the commercial preparations

Wright To emphasize this still further I might point out that initially some of the commercially prepared non rabbit brain preparations have almost overlapping curves with rabbit brain thromboplastins but when a level of say 35 or 40 seconds is reached something like an escape mechanism seems to occur with the curve of the former rising more rapidly than that of the latter sometimes up to 65 or 70 seconds On the other hand the thromboplastin used routinely by Dr Overman may show a rise of only 3 to 10 seconds during the same period of observation The excessive response alarms those unaware of this phenomenon This is an interesting observation and one which probably Dr Mann explains on the basis of his co thromboplastin" From a practical point of view it means that the clinician has to know all the details of any procedure used for the control of anticoagulation therapy

Mann On the basis of our experience with the different sensitivities of different thromboplastins to the effect of dicumarol we are entirely convinced of the correctness of your observation We are equally convinced that these sharp changes which are shown by a sensitive thromboplastin are of very definite significance

Barker In our work with tromexan where we noted rather wide fluctuations of the prothrombin time particularly during the first two weeks of treatment I was not concerned as much by the peaks which are usually transient as I was by the valleys where the prothrombin time dropped below the therapeutic range During such periods theoretically at least patients may not be adequately protected against thrombosis

Wright Of course that has been one of the questions with heparin in intermittent dosage and yet from a practical point of

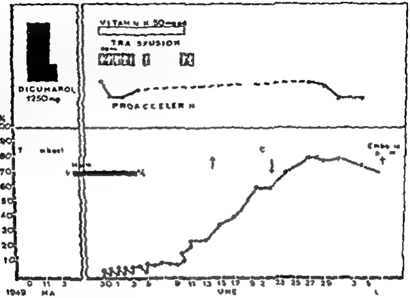

Figure 24 Dicumarol intoxication

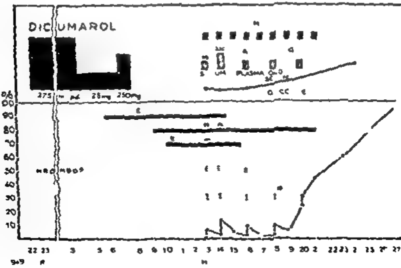

Figure 25 Dicumarol intoxication

We have found that dicumarol treatment is followed by a decrease in both prothrombin and proconvertin Proconvertin usually is decreased to a lower level Proaccelerin is unaltered (Figure 26)

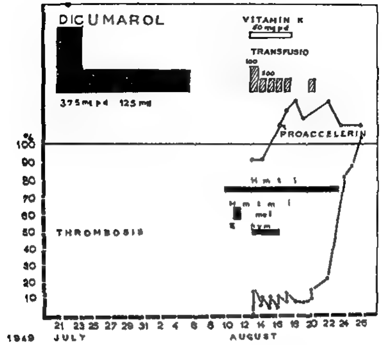

FIGURE 23 Dicumarol intoxication The curve gives the P & P value The upper curve gives the proaccelerin value See text

We used only 50 mg a day which is a very low amount in such a severe case At other times we have given up to 1 gm in twenty four hours in divided doses When the intoxication is not too severe some effect is then observed

The accelerator factor (factor V or proaccelerin) is not affected in severe dicumarol intoxication as shown in Figure 23 and therefore the effect of fresh plasma (normal in proaccelerin content) and that of stored plasma (low in proaccelerin) is the same

The next case (Figure 24) illustrates that it may be dangerous to bring the prothrombin level too rapidly up to normal by K_1 oxide or other treatment The patient had a fatal pulmonary embolism when the prothrombin level had been about normal for some days

Figure 25 *shows a similar case of severe intoxication* it also demonstrates that hematuria may persist until the prothrombin level has risen to about 40 per cent That transfusion of serum fresh or stored also increases the P & P value is shown This effect is caused by the fact that serum contains proconvertin

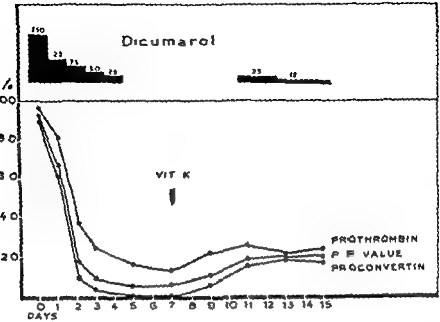

FIGURE 27 The effect of dicumarol on prothrombin and proconvertin

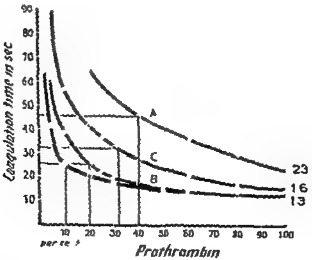

FIGURE 28 The relation between prothrombin activity and clotting time for different thromboplastins (A and C human brain B rabbit brain)

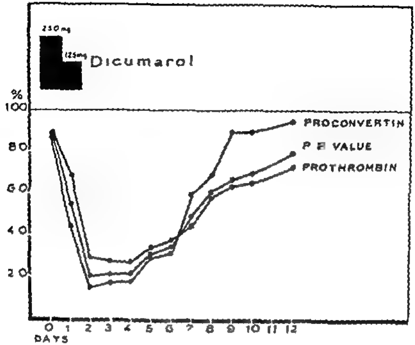

FIGURE 26 The effect of dicumarol on prothrombin and proconvertin

In some cases proconvertin may disappear completely while prothrombin is still in the therapeutic range (Figure 27)

I want to make a short comment concerning the discussion of the value of different types of thromboplastin The curve showing the relation between prothrombin activity and clotting time (Figure 28) varies with the type of thromboplastin used By the use of a very active thromboplastin (rabbit thromboplastin curves B) there will be a certain critical point on the curve where the prothrombin time suddenly increases rapidly to unmeasurable values Other types of thromboplastin (human brain thromboplastin curves A and C) show a more even slope of the curve The two types of rabbit thromboplastin (curves B) illustrate the fact that in spite of both extracts giving the same normal prothrombin time of 13 seconds we have no guarantee that identical prothrombin times in the lower concentration range of prothrombin mean the same prothrombin activity

Best May we know how proconvertin and convertin are estimated?

Owren I shall come back to that question later

most of the major bleeding comes from operative wounds I think that the type of operative wound is a more important factor than the age of the patient for example whether it is a wide open wound such as a transurethral prostatectomy or whether it is a tightly closed wound such as a herniorrhaphy The vaginal wound after total hysterectomy seems to be particularly prone to develop granulation tissue and I think the bleeding develops in these patients because the granulation tissue is present rather than because the blood vessels are old and sclerotic

Alexander May I ask a question which has troubled us regarding the protracted use of the dicumarol derivatives and also phenylindandione Have you any observations Dr Barker that after long use of any of these agents in some instances the prothrombin time may fail to return to normal for weeks if not months after discontinuance of the drug?

Barker I would say no but my experience with long term anticoagulant therapy is smaller than that of others in this group

Brambel During six years of studying anticoagulant therapy we found that the prothrombin time promptly returns to normal regardless of how long the anticoagulant agents are administered Furthermore some of the patients died and the autopsies revealed no pathology* that could be related to anticoagulants

Wright That is our experience too We are now compiling figures on more than 200 patients who have been on anticoagulants from one month to six years totaling approximately 220 patient years of therapy Our experience parallels Dr Brambel's namely that a person may have slightly different requirements from time to time but at the end of three or four years he is usually maintained on essentially the same dosage as he was started on

It may be of interest to point out that we have studied two individuals who had rheumatic heart disease with auricular fibrillation and who were on anticoagulants for three years without emboli One patient had previously had fourteen emboli the other had had twenty nine of which were in the ten days before the patient received anticoagulants They both stopped taking anticoagulants on their own initiative and against our advice Within thirty days after cessation of treatment both patients had emboli one a saddle embolus at the aortic bifurcation and the other a cerebral embolus Our records contain many similar instances

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* EDITOR'S NOTE Pathologists are always annoyed by this use of the term Pathology means the science of disease In the above sentence *morphologic alterations* should be substituted for *pathology*

Allen My remarks concern a problem which was touched upon earlier namely, the effectiveness of transfusions in treating hypoprothrombinemia My experience in this respect relates to the care of surgical patients In some instances we administer a liter of plasma to patients with hypoprothrombinemia and under such circumstances the prothrombin is raised sufficiently to control a bleeding problem

Wright Your point I presume is that too small a transfusion is often given to patients with hypoprothrombinemia

Allen Yes and that is why I think Dr Seegers point is well taken that if prothrombin could be prepared – it would even be better if it were human prothrombin – one would have a use for such a product in dealing with postoperative hemorrhage from prothrombin deficiency This type of bleeding may be exsanguinary in an hour or two if it is not controlled

Warner Are the older age groups on anticoagulant therapy more apt to bleed than younger individuals?

Barker In my opinion the answer is yes

Wright I think I would say yes too but I should like to qualify it by saying that the older the person, the more apt he is to have an occult carcinoma ulcerative colitis or other conditions predisposing to easy bleeding such as blood vessel damage in the kidney and urinary bladder Perhaps the patient has been losing small amounts of blood for a long time but never in sufficient quantity to be clinically recognized If all predisposing conditions could be excluded I do not know that there would be very much difference between the younger group and the older group In our experience many of the serious hemorrhages arise from the stage being set by other disease processes

Flynn Philosophically this is expressed by the principle of multiple causation It illustrates the rather obvious but sometimes overlooked principle that an effect is usually the result of several conditions and that the totality of all the conditions leading to a certain result are the cause of a phenomenon Thus a patient with an active peptic ulcer and marked arteriolosclerosis may have severe gastrointestinal bleeding due to inability of the sclerotic vessel to contract In this case the arteriolosclerosis and the peptic ulcer although independent conditions that are unrelated in themselves will nevertheless when combined at the right time in the right place have a causal relationship to gastrointestinal bleeding

Barker Actually we encounter major bleeding rather rarely We use anticoagulant therapy in a good many surgical patients and

most of the major bleeding comes from operative wounds I think that the type of operative wound is a more important factor than the age of the patient for example whether it [redacted] wide open wound such as [redacted] transurethral prostatectomy or whether it is a tightly closed wound such as a herniorrhaphy The vaginal wound after total hysterectomy seems to be particularly prone to develop granulation tissue and I think the bleeding develops in these patients because the granulation tissue [redacted] present rather than be cause the blood vessels are old and sclerotic

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Alexander We have seen a few instances where protracted use of phenylindandione resulted in the prothrombin time remaining elevated despite discontinuance of the drug

Blaustein I have not had the experience that the prothrombin time remains prolonged after phenylindandione is stopped nor do I know of anyone else who has I have heard of a case on dicumarol therapy which Dr Shapiro* had where the prothrombin time remained prolonged for two months after the drug had been stopped

Wright It seems to me that one has to be very certain that an intervening hepatitis or some more obscure change in the liver has not developed in these cases Such changes may produce hypo prothrombinemia not related to the drug under consideration

Brambel We have done a study on a population of about five hundred outpatients for the last six or seven years and have not seen a single instance of refractory hypoprothrombinemia following cessation of anticoagulation therapy

Wright We seem to be discussing two problems one is a failure of the prothrombin to return to normal after anticoagulant therapy has been stopped the other is an increased sensitivity developing to the anticoagulant during therapy I have seen cases of apparent sensitivity i e, marked prolongation of the prothrombin time following therapeutic doses which could be explained on the basis of hepatitis

Alexander Dr Link at one time you said that in your early work you suspected that some of the sulfonated polysaccharides used for anticoagulant therapy would have untoward reactions?

Link That was based on observations of how cats behaved under dosage They did not look good to me Furthermore an examination was made of their kidneys by a pathologist and it was shown that they had some kidney damage As a result we concentrated on dicumarol

Wright Dr Link you did not actually work with the compound paritol did you?

Link No not paritol but we had compounds in that class

Wright Yes I understand that but I just wanted to be certain because it makes some difference in the conclusion

Barker Did you do some work with treburon?

Link Yes The mother product had been made in my laboratory from pectin back in 1936

Best Was it the same one?

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* Personal communication

Link That is debatable I asked Dr Overman how many sulfuric radicals it had Roche s product has 2 and he tells me that ours had about 1 5 There is some variation there But they all on the basis of our work looked toxic Now that was a farmer s intuition Dr Best Don t ask me beyond that

Seegers I am very much intrigued by the possibility of doing an experiment in which a dog receiving dicumarol is given purified prothrombin over a protracted period of time It might be worth the investment for it would at least produce experimental proconvertin deficiency if I understood Dr Owren correctly that dicumarol lowers both prothrombin and proconvertin If we could assume that the prothrombin was free of proconvertin we would be supplying the animal with prothrombin and we should have only a proconvertin deficiency And if with a proconvertin deficiency we did not get a bleeding tendency that would be a good indication that one ought to look for a drug that would only lower proconvertin and leave the prothrombin where it normally is Then there would not be the problem of the physician giving an overdose

Brinkhous Do you make prothrombin preparations which are proconvertin free?

Seegers I hope they are but I have no way of knowing for sure You can never say anything is absolutely free of anything and particularly in this instance I don t know how to go about finding out

Wright Then your experiment would not quite be valid unless you had more information on that would it?

Seegers I think all of our experiments are successive approximations to the truth I have an idea that our methods can produce prothrombin which is homogeneous in the ultracentrifuge The electrophoretic analyses also indicate homogeneity and the whole mobility pattern changes when the product is changed to thrombin in a 25 per cent sodium citrate solution If there is proconvertin in our most active prothrombin preparations it almost certainly has to be there in small proportions But there is always an opening for someone to say that those small concentrations are getting us into trouble on critical points

Owren It would be easy to control the content of proconvertin in a preparation of prothrombin by transfusion to a patient with proconvertin deficiency It could be seen whether the proconvertin level increased after transfusion thereby shortening the clotting time We also have methods for specifically determining prothrombin and proconvertin

Mann I know of some evidence that at least one of Dr Seegers preparations was completely free of conversion factors Dr C A Owen found that this preparation would form no thrombin in the presence of thromboplastin and calcium and very little when adsorbed plasma was added as a source of the labile prothrombin conversion factor When however it was mixed in appropriate proportions with normal plasma it promptly formed the expected increment of thrombin (3)

Wright Do you consider that a valid experiment Dr Seegers?

Seegers Well I imagine that it is

Blaustein Before the meeting Dr Barker and I were talking about the problem that has concerned me most short term anticoagulation therapy Phenylindandione is used in some cases of short term therapy but one must be aware of the fact that patients may be resistant to this drug This means that in a postpartum case with phlebitis it may take a few days to find out whether phenylindandione is effective in lowering the prothrombin level If it does not prove effective more harm than good may have been done I think Dr Barker pointed out that the same problem exists with tromexan

Statistically we have now found 3 75 per cent of cases are resistant to phenylindandione This percentage was much larger at one time It is hard to give a figure that has real meaning because it changes as the series becomes larger At one time the figure for resistance was almost 12 per cent Since our original publication on phenylindandione several others have appeared recently one by Preston and Thompson of Northwestern University In seventy four cases treated with phenylindandione they report nearly the same results we obtained They describe a similar percentage of resistant cases but believe that larger initial doses or larger maintenance doses would have obviated this However we have demonstrated that this is by no means true since we have given doses as high as 500 mg and have not been able to lower the prothrombin to a clinically effective level Furthermore this has been shown at the Goldwater Hospital too where Dr Murray Weiner gaves doses of 500 and 600 mg initially and in some instances was not able to obtain adequate levels *

Preston and Thompson report three cases of bleeding In one of them there were petechiae The patient however was on quinidine and it is well known that quinidine can cause thrombocytopenia

* Personal communication

hence I doubt that this case can be attributed unequivocally to the phenylindandione To summarize Preston and Thompson think phenylindandione is a good anticoagulant except for short term therapy I believe that phenylindandione is effective anticoagulant but that its use for short term therapy requires qualification

Wright Dr Blaustein what has been your experience as to the relative predictability of the anticoagulant effects of tromexan phenylindandione and dicumarol?

Blaustein In my laboratory blood for prothrombin times comes from a large number of physicians and the results are most erratic Those clinicians who take anticoagulant therapy seriously seem to get good results with any anticoagulant those who use it haphazardly get poor results

Several cardiologists in one of the New Jersey hospitals use tromexan and get consistently good results I have not been able to get them to use phenylindandione since tromexan has proved so satisfactory Where results have been poor with tromexan I have been called in consultation to see if the laboratory was at fault I found that the greatest difficulty comes from physicians giving adequate initial doses but improper maintenance doses Less commonly they give an adequate initial dose but too much of a maintenance dose In my experience tromexan in careful hands is an excellent anticoagulant but in poor hands it is a hazard The same thing applies to phenylindandione As far as dicumarol is concerned I know of many men who use it However I do get dicumarolized plasmas in my laboratory which often have such prolonged prothrombin times that the clotting times can be described as infinity

In the type of work we do it is difficult to determine the relative values of the various anticoagulants There is also the problem of what type of thromboplastin is used

Wright Those of you who were present at the first Conference heard me make a plea for a serious endeavor to standardize thromboplastin At that time it seemed as though my plea was premature at least some of the members thought so However we are not very much further ahead five years later than we were then It remains one of the major problems in the whole question of anticoagulant therapy Dr Overman has been working with different thromboplastins for several years and I shall have him comment on his findings

Overman As Dr Blaustein inferred there are wide variations in activity among the various commercial thromboplastin preparations Also it is easy to show wide variations in activity obtained

from the same batch of thromboplastin just by slight variations in the technique used in preparing the thromboplastin suspension This was one of the reasons we started working on the chemical purification and isolation of thromboplastin several years ago In the course of this work we isolated a fraction from the phospholipid fraction of thromboplastin preparations which inhibited the coagulation of blood (4) One of our objectives has been to isolate this inhibitor in a pure state so that by studying its activity we might be able to clear up some of the problems concerning thromboplastin activity The fraction isolated from brain and lung thromboplastin inhibits the activity of thromboplastin at certain concentrations When this material is added to the protein fraction remaining after the isolation of the inhibitor thromboplastin activity is reconstituted We consider the concentration of these two components the inhibitor and the protein to be very important in determining the activity of thromboplastin preparations

As we previously reported the inhibitor was present in the inositol phosphatide fraction of brain and lung thromboplastin A fraction was also isolated from soybean phosphatides which inhibited the action of thromboplastin By further work we have been able to show that the material isolated from brain and lung thromboplastin is very similar in chemical and biological properties to the fraction isolated from soybeans The active fractions now have been purified to the extent that they do not contain sulfur This fact is further proof that this inhibitor is not heparin This is also substantiated by its biological activity which in most aspects is different from that of heparin Heparin has antithrombic activity which we have been unable to demonstrate with the thromboplastin inhibitor This has also been shown by Dr Tocantins with the lipid antithromboplastin isolated from brain which he has been studying Heparin prolongs the prothrombin time *in vitro* when Russell viper venom is used as a source of thromboplastin The phosphatide thromboplastin inhibitor does not prolong the prothrombin time when Russell viper venom is used In addition protamine counteracts the anticoagulant activity of heparin whereas it has no action when it is added to a system containing the phosphatide inhibitor

These problems are all related to the fundamental subject of what is essential for thromboplastin activity Whether thromboplastin is absolutely essential for the activation of prothrombin or not is another question particularly in view of the observation that calcium is not necessary for the activation of prothrombin to thrombin in diluted oxalated plasma systems (5) which was subsequently

confirmed by Seegers (6) using purified prothrombin It might be that the accelerator or conversion factors of Drs Quick Owren Fantl and Seegers act only after prothrombin is activated by some means the presence of thromboplastin being one such mechanism These problems can be definitely solved only by working with purified systems

Wright I should like to refer again to a remark Dr Mann made some time back He expressed the belief that the high peaks of prothrombin time occurring with certain thromboplastin preparations are significant Actually from the point of view of bleeding we have found that under certain conditions they are of little significance Some thromboplastin preparations produce a fairly satisfactory curve up to the thirty second range but then show a very high spike for ranges above this level This is particularly apt to occur with tromexan to a lesser degree with dicumarol While it is true that these results may be significant from the standpoint of fundamentals it is nevertheless confusing to a physician to receive a report of a prothrombin time of 60 to 70 seconds when a more standardized thromboplastin would reveal one of only 34 or 35 seconds

Brambel Taking all the data that have been assembled to date one is impressed with the fact that several different thromboplastins have given the same result Your group uses one thromboplastin Dr Barker's group uses another and we use still another yet our results with respect to thromboembolism are fairly comparable

Wright We understand the tools we work with when we discuss such a problem in a team like this We have not succeeded in protecting the physician who is not working in a group with trained clinical pathologists or is not an internist with special interests in this field It distresses me as it must all of you that we have not been able to do this

Alexander There is one point about technique which needs to be emphasized to the practicing physician In the procurement of a sample of blood for the determination of prothrombin activity by the one stage method every effort must be made to obtain the blood meticulously from the point of view of venipuncture Probing around in the tissues before drawing the blood or allowing the blood to stand before it is put into anticoagulant or contaminating the needle and syringe by tissue juice will very often give a distorted and erroneous value As Dr Blaustein has pointed out he is the recipient of samples that are sent in to him from many physicians and the question of whether proper technique was em

ployed in obtaining the sample is of the utmost importance at least in accounting in part for certain bizarre results

Wright There is where a laboratory worker in Dr Blaustein s position can diplomatically constitute an educational spearhead

Brinkhous Conversely it might be pointed out that blood drawn too carefully or prothrombin time determinations done too quickly also give what seem to be erroneous results Under these circum stances a prolonged prothrombin time is obtained

Wright How can something be done too carefully in science Dr Brinkhous?

Brinkhous I am not talking about science but about the labora tory control of anticoagulant therapy This point was well shown by Dr Langdell (7)

Olwin We recently have been using tromexan in our antico agulant therapy and it seems of interest to present our work inas much as it is somewhat divergent from Dr Barker s We have studied tromexan mainly from its effect on the one stage and two stage prothrombin determinations We have also studied antithrom bin as measured by Dr Seegers method and Ac globulin also by his method except that we have substituted stored plasma as a source of prothrombin in place of purified prothrombin I shall not report on the latter two factors at this time

It seems reasonable to me at least that all new prothrombin reducing anticoagulants should be compared to dicumarol Figure 29 illustrates the course of a patient on long term therapy with dicumarol The two stage method is the Iowa two stage assay modi fied by Dr Seegers that [illegible] Ac globulin has been added The one stage method is the whole plasma technique using rabbit brain the method Dr Quick has outlined It can be seen that given a reasonably uniform dose of dicumarol the patient by the two stage method shows a fairly stable prothrombin whereas by the one stage method the prothrombin varies widely If the patient had been treated with a larger lose of dicumarol on May 31 (Figure 29) his prothrombin very likely would have dropped to a bleeding level With the dicumarol given daily in uniform doses the pro thrombin is maintained at a fairly consistent therapeutic level This represents the course in perhaps 50 to 60 per cent of our patients on dicumarol

Warner It appears that the prothrombin particularly by the *one* stage but also by the two stage method went up before the dicumarol was discontinued

Olwin I had not noticed it before but I believe there is an error

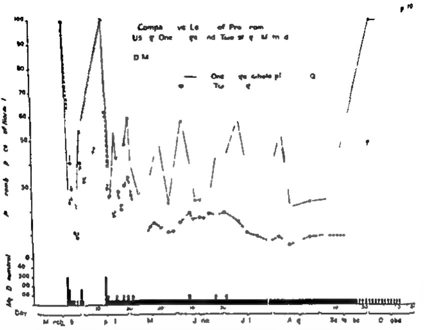

FIGURE 29 : With the patient on a relatively uniform daily dosage of dicumarol the prothrombin level as measured by the two-stage method remains quite stable The one-stage test on the other hand fluctuates widely Were the dosage of dicumarol being controlled by the latter the prothrombin might at several points have been reduced to a bleeding level

in the chart The next to the last one stage point should be moved a bit to the right and the one stage point corresponding to the third to the last two stage point has been omitted I apologize for the mistake

Unlike dicumarol with tromexan therapy our experience has been that the one stage and two stage tests more nearly parallel each other Figure 30 represents the course of a patient receiving tromexan in single (undivided) daily doses The tests were run under the same conditions as in Figure 29 with the exception that in the one stage test we used solu plastin a commercial horse tissue extract We have tested various batches of solu plastin and have found it to be the most stable and the most uniform in its reactivity of all the commercial thromboplastin preparations We test each batch as it comes along and a curve is made weekly with normal plasma If it does not meet the requirements of 10 to 11 seconds for normal whole plasma it is rejected We now use it routinely in place of the rabbit brain which we formerly prepared in our own laboratory

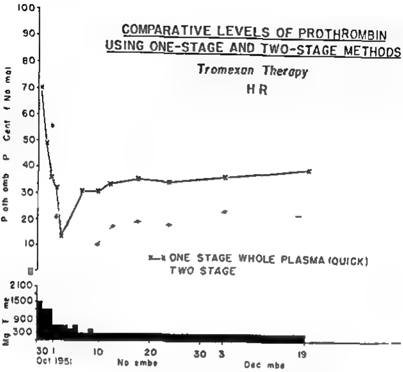

FIGURE 30 In patients receiving tromexan the one stage (using whole plasma) and two stage methods more nearly parallel each other in their measure of prothrombin than is true when dicumarol is used This provides for a more accurately controlled therapeutic bracket and hence a greater safety from both the danger of too much clotting and of too much bleeding

Flynn Is it valid to say that the tests were more parallel with tromexan since when you changed the thromboplastin you changed the conditions of the experiment?

Olwin That s a good point The one stage levels represented in Figure 29 were attained with acetone dried rabbit brain However we shifted to the use of solu plastin two years ago Furthermore we used dicumarol routinely until six months ago We have been using tromexan for the last six months Figure 29 is representative of most of our patients receiving dicumarol whether rabbit brain or horse tissue extract (solu plastin) is used as thromboplastin In most of our cases perhaps 90 per cent the two tests were fairly parallel when tromexan was given This was less often true when patients received dicumarol When tromexan is given in interrupted doses that is a large dose one day and none the following day when the prothrombin may be in the therapeutic bracket or slightly below it the variation in the prothrombin levels will be wide It

must be remembered that the recovery period as well as the induction period for tromexan is less than that for dicumarol and if the prothrombin is to be maintained at a uniform level the drug must be given at sufficiently frequent intervals It has been our practice not to stop the tromexan when a low level is obtained We reduce the dose but continue to give it daily Then the type of situation shown in Figure 30 usually holds The prothrombin levels off early within the desired therapeutic bracket and remains there once the individual tolerance is determined The cases in which this has not been true have for the most part been those in which we were faint hearted and dared not continue tromexan when the prothrombin dropped to the lower limit of the therapeutic bracket (i e 10 per cent as measured by the two stage method) I do not mean to say that with tromexan we never get to the point where we omit the drug for a day or so but in general the principle of regular daily doses is followed

As I mentioned in the neighborhood of 90 per cent of our cases follow the pattern found in Figure 30 This patient had a somewhat lowered original prothrombin so we dropped to 1500 mg instead of the usual 1800 as an initial dose In an occasional case we give 2100 mg initially On the second day we usually drop to 1200 and the third day to 900 from then on the prothrombin level determines the amount of tromexan given The daily dosage varies in individual patients from 300 to 900 mg An occasional patient will require divided doses half of the daily dose in the morning and half in the evening

Thus in our experience tromexan gives an earlier and a more uniformly controlled patient than does dicumarol secondly the one stage test more nearly parallels the two stage test in patients given tromexan than in those given dicumarol This may be of rather important practical consideration inasmuch as most laboratories in the country depend upon some form of the one stage test to control their anticoagulant therapy We believe that the safety factor is considerably greater in tromexan treated patients than in those receiving dicumarol, provided of course careful attention is given to the administration of the drug Thirdly it would appear that fewer factors are affected with tromexan therapy than with dicumarol Certainly the variations in these two tests would indicate that there is some difference in the effect of tromexan and dicumarol on the coagulation and/or anticoagulation factors

We have used Dr Link s anticoagulant No 63 on a few patients Figure 31 shows the curve obtained for one patient to whom 200

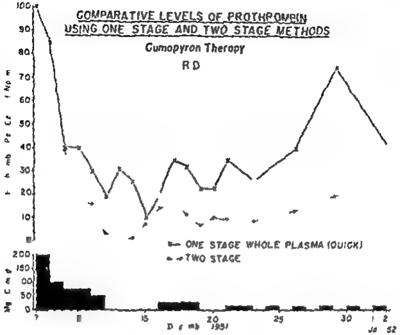

FIGURE 31 This represents the prothrombin levels of a patient receiving cumopyrin Dr Link s compound No 63 It more nearly resembl s dicumarol in its effects than does tromexan It is less potent than either drug and like dicumarol, does not affect the one stage and two-stage prothrombin levels in as uniform a fashion as does tromexan

mg of No 63 was given which may have been too large an original dose The patient s prothrombin with the two stage test was fairly uniform and in a therapeutic bracket On December 12 13 and 14 it is definitely a little lower than is desirable although the one stage shows a satisfactory margin of safety However with interrupted doses of No 63 the prothrombin was maintained at a more desirable level The one stage is not quite as uniform as we found in the case of the tromexan patients Whether or not this Figure is representative of a large series of cases cannot be decided as we have not had enough experience with No 63

Tocantins My remarks are a supplement to what Dr Overman had to say earlier Even in our early crude preparations of lipid antithromboplastin no evidence of sulfur was found That is one of the reasons why we believed very strongly at the time that we were not dealing with heparin We could not demonstrate any antithrombin activity in our antithromboplastin preparations We never found that protamine inactivated or offset the anticoagulant effect

of the lipid inhibitor And even when we added heparin to the preparations of the lipid inhibitor and then tested the effect of the protamine we could account for all of the effect of the protamine on the basis of the amount of heparin that had been added to the mixture Thus we felt quite certain that we were not dealing with heparin and everything that has come up since then has helped to confirm that impression

Cronkite Dr Tocantins when you added the protamine was it in a platelet free system?

Tocantins Yes We took a purified suspension of the antithromboplastin and added a given amount of heparin in it in turn adding that mixture to the platelet poor plasma When we did a protamine titration on that plasma we could account for all the heparin present by the amount of protamine required to bring the clotting time back to that of the control the control being plasma and the lipid inhibitor only

Cronkite The reason I ask that is that protamine in very small amounts *in vitro* will cause the platelets literally to explode and I know that you had the idea that perhaps the platelets and your lipid antithromboplastin were mutually antagonistic

Tocantins Yes This was done in platelet poor plasma It was done in whole blood or even in plasma containing more than 4000 platelets or so per cubic milliliter

We have not made the progress that we had anticipated in purifying the lipid antithromboplastin We have made a few studies on the distribution of this material in the body The brain contains the greatest amount but it is found generally in all tissues of the body The second highest concentration of the lipid inhibitor is found in the adrenals that is in terms of activity per dry weight of the organ Why that is I dont know but it is well known that the adrenals contain a large amount of lipid material

We have not done any more extensive work in an attempt to use this inhibitor for anticoagulant therapy and that is simply because we have not purified it to the point that we have freed it from clot promoting substances However we have made further trials of the inhibitor on animals and even with our crude preparations we are satisfied that our original observations namely that the inhibitor when injected intravenously has a lasting quality and is entirely free of side effects were essentially correct

Flynn Dr Coon Dr Vassar and I prepared the lipid inhibitor according to Dr Tocantins methods and found that we could give relatively large doses of this material to dogs (8) Furthermore the

animals did not develop apparent sensitivity to the material, despite the fact that some of the dogs had repeated doses over many months A few of the animals were sacrificed but no significant morphologic alterations were demonstrated

Plasma containing lipid inhibitor gave low values for prothrombin and factor V In the case of prothrombin the inhibitor affected both the yield of thrombin and the rate of thrombin evolution If we added factor V to the lipid treated plasma we obtained an appreciable increase in the prothrombin activity an increase over and above what could be accounted for by the trace contaminant of prothrombin in the factor V Our first hypothesis was that the lipid inhibitor was antagonistic to factor V We hoped that if factor V really inactivated the lipid inhibitor the reaction might require enough time so that we would get a different result when a preliminary incubation of the reagents preceded the addition of plasma than when all reagents were added simultaneously Accordingly we prepared mixtures of lipid inhibitor and factor V and let them stand for various times before adding the plasma Essentially the same result however was obtained regardless of whether the plasma was added one minute or thirty minutes later If a preliminary mixing had affected the plasma prothrombin activity we would have had evidence that a reaction occurred between the factor V and lipid inhibitor but the result we obtained neither proved nor disproved our hypothesis

We then investigated other possibilities by using purified reagents and doing a preliminary mixing of the lipid inhibitor with factors such as Ca^{++} thromboplastin etc Again preliminary incubation did not affect the result It is possible of course that the reaction of the lipid inhibitor with whatever factor or factors are involved occurs so rapidly as to be unmeasurable but this seems improbable to us Indeed we are inclined to believe that the addition of factor V rather than antagonizing the lipid inhibitor may merely accelerate the conversion of the residual prothrombin which is not directly or indirectly affected by the lipid inhibitor

Another observation we made was that the lipid inhibitor is more effective *in vivo* than *in vitro* We think this may represent a matter of dispersion When the lipid inhibitor is mixed with plasma *in vitro* a milky emulsion is obtained but *in vivo* the plasma remains almost water clear Along this line Dr Tocantins has done some work with a supersonic vibrator which he may care to discuss

Intuitively we believe that the lipid inhibitor may prove to be of great importance Even if it is without clinical significance it might

be a useful tool in the analysis of the interaction of the many variables involved in thrombin formation

Tocantins We have been using a supersonic vibrator for about three years in an attempt to raise the potency of the lipid antithromboplastin and it is very effective A solution that is milky and of the consistency almost of heavy cream for example a 5 per cent solution of brain lipid antithromboplastin when exposed to the supersonic vibrator for about ten minutes becomes as clear as water and its potency goes up ten to twenty fold

Wright If it is allowed to stand will it revert back to the milky state?

Tocantins If it is allowed to stand particularly in the cold it will slowly go back to its original state A per cent emulsion of the lipid antithromboplastin emulsified with a hand homogenizer is thick and creamy After exposure to the supersonic vibrator it becomes perfectly clear clear enough to read newspaper print through and it is a strong anticoagulant If that same clear lipid emulsion is frozen and thawed it becomes white and creamy and its potency as an anticoagulant is much reduced Exposed to the supersonic vibrator again it becomes clear and regains its high potency You can do that back and forth as many times as you choose

Ferguson Chiefly for my own information does anybody have recent knowledge concerning heparin substitutes other than those we have considered at these Conferences? I refer specifically to thrombocide and to sulfonated dextran Last year (9) we commented on our experiments with thrombocide Has this drug been put on the American market?

Wright That is one of the preparations that produces alopecia so I think it is very unlikely that it has been used much in this country

de Nicola I tried some experiments with thrombocide although I had already heard that it produces alopecia (10) In connection with its anticoagulant activity we had the impression that the duration of the effect with the suggested dosage was too short Figure 32 shows that by means of injecting 200 300 or 400 mg of thrombocide an anticoagulant activity is obtained for not more than about three hours Doses needed to produce a continued effect over a period of twenty four hours are too toxic (11 12)

Flynn Did you notice any fibrinolytic activity? Halse (13) I believe reported that thrombocide dissolves clots

de Nicola Some experiments done in Vienna showed that throm

animals did not develop apparent sensitivity to the material despite the fact that some of the dogs had repeated doses over many months A few of the animals were sacrificed but no significant morphologic alterations were demonstrated

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Tocantins As far as I know they are not

Overman We have done some preliminary work with the phosphatide inhibitor isolated from soybeans and when administered by mouth it was not biologically active in prolonging the coagulation time

Barker We tried to administer treburon sublingually but were unable to demonstrate measurable effect on coagulation

Wright It might be appropriate to mention that we have attempted to reproduce the work of Latwins and his group (17) on the sublingual or buccal administration of heparin Unfortunately it has now been clearly demonstrated that this method is not very effective It appears that their erroneous conclusions arose because of the technique used in doing the clotting time tests These tests were apparently done in such a way as to shake the tube and this will produce a prolongation in the clotting time A prolongation was not encountered when the clotting time tests were carefully controlled

Tremont Smith Is there any possibility that your material Dr Tocantins could have an antioxidant activity?

Tocantins There is a possibility We have never tested it for that property It is very poorly oxidized

Flynn Dr Barker in your presentation you constantly referred to patients who were sensitive to anticoagulant therapy and to those who were resistant Do the terms sensitive and resistant as you used them refer to the lower and upper limits of the range of expected variations or do they have a different connotation?

Barker I am not sure just how to answer because it is hard to define a normal and an abnormal response Most of the variations in response among different individuals are probably best classified as variations within a normal range of response to a drug However patients who have had a very recent surgical operation are usually somewhat sensitive to the coumarin compounds This sensitivity could be the result of transient dietary deficiency anesthesia or blood loss Perhaps such sensitivity should not be considered to be within the normal range

Wright Do you concede that there are diseases which produce additional variability over and above that?

Barker There are many known conditions that usually produce additional variability for example frank nutritional deficiency hepatic disease renal insufficiency and certain blood dyscrasias Some patients with active thrombosis seem to be unusually resistant But there are unknown factors apparently which produce

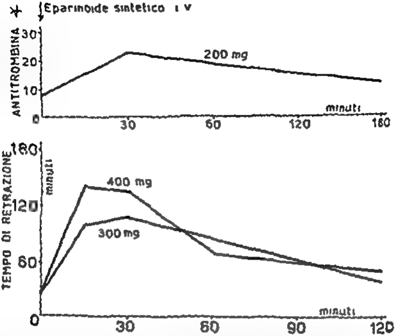

FIGURE 32 Evaluation of the anticoagulant effect of thrombocide The upper curve represents plasma plus thrombin at various concentrations (so-called "heparin antithrombin") according to the *technique described by Koller and Fritschy* (14) the lower curve is the retraction time according to the technique of Hirschboek (15) The "antithrombin angle" used in the upper curve is the method of Wanner (16) Reprinted by permission from de Nicola I Trattamento delle ipereparinemie con protamina intramuscolare ad effeto prolungato *Progr med Napoli 7 300 (1951)*

bocide has no fibrinolytic activity That was true not only for thrombocide but also for heparin I think there is no definite proof of such fibrinolytic activity of heparin and heparin like substances Furthermore at the last meeting of the European International Society of Hematology in Rome as I think Dr Owren will remember stress was laid on the lack of proof for fibrinolytic activity of such heparin like substances

Alexander Dr Tocantins have you any information as to the fraction of the plasma in which the antithromboplastic material is present?

Tocantins Yes We presented the data on that to this Conference three years ago The lipid inhibitor can be found in the Cohn fraction IV 1 and IV 3 4 particularly in high concentration in IV-3 4 Very little is found in the other Cohn plasma fractions

Alexander One other question are any of these compounds effective by mouth?

cules and greater solubility might more readily be eliminated through the kidneys

Blaustein In our early work we found that rats appeared to become resistant to dicumarol tromexan and P I D even when on a vitamin K deficient diet It is true that at the time the rats became resistant the conditions of the experiment had changed since originally the anticoagulants were supplied in a slightly alkaline solution of drinking water but later the anticoagulants were given in the diet

Alexander Could it not have been the alkali? The alkali certainly renders the dicumarol preparation far more soluble than ordinarily As a matter of fact it has been established that the alkali opens up the lactone ring increasing the solubility

Warner In those rats were the prothrombin measurements done by the one stage method?

Blaustein Yes

Warner Several years back Dr Boyd (18) in our laboratory studied a number of rats given dicumarol It was administered by stomach tube so that the dose was standard A majority of the animals did develop a progressive resistance to the effect of dicumarol as measured by repeated two-stage prothrombin determinations over a period of several weeks Eventually the dose was raised to several times what had been an effective dose to start with There would be a day here and there when the prothrombin was low that they would not receive any but it was a continuous experiment And they did develop a resistance it seems

Overman In much of our work we have administered dicumarol by stomach tube but I have never noted any marked difference in response after repeated doses over a period of several weeks or months Dr Blaustein you stated that originally you gave the anticoagulant in slightly alkaline solution It is likely that not much of the dicumarol dissolved since a pH of approximately 10 is necessary to keep dicumarol in solution It might be that originally the rats were not getting any dicumarol which would explain your results

With tromexan one has to give very large amounts before an effect on the prothrombin time is detected in the rat The rat is more resistant to tromexan than dicumarol We gave 100 mg of tromexan daily to rats for a period of five days without any change in the prothrombin time

Blaustein Despite the low alkalinity of our solution the prothrombin times were prolonged so the animals were getting some of the dicumarol

relative sensitivity and relative resistance, these are unpredictable at present and until they are known the variations produced by them have to be classified as within the range of normal responses among different people

Warner In some of the charts that you presented earlier, there seemed to be little if any effect of the menadione compound in counteracting the anticoagulants action and I understood you to say that the individual who seemed conspicuously hypersensitive to those anticoagulants was one who was rather likely to be beneficially affected by menadione Then also there was the question of the protracted resistance to subsequent administration of the anticoagulants after the K_1 refractory period I was wondering at the time whether there was possibly any correlation in the sensitivity versus conspicuous resistance to these anticoagulants in people who would be candidates for K deficiency or high K intake The ones that are peculiarly hypersensitive the postoperative patients particularly — and it depends upon the type of operation — may be cases that are on the ragged edge of a K deficiency It might tie up with the more effective response to the ordinary water soluble compounds

Barker We have had that very same idea but only as the result of reasoning from the data We have not been able to prove it In other words we have felt that the postoperative patient during the immediate postoperative period might have a submeasurable deficiency of vitamin K and therefore an exaggerated response to a coumarin anticoagulant

Wright We have reported that some individuals after receiving intestinal sterilizing antibiotics are over responsive to hypoprothrombinemic producing anticoagulants despite the fact that the same doses did not produce this effect before the antibiotics were administered Our explanation has been a purely hypothetical one as follows The destruction of the bacteria in the gut interferes with the production of K and therefore K no longer acts as an adequate buffer substance against the anticoagulant This is a question which deserves investigation The phenomenon has occurred in a number of our patients It is characterized by a very sharp rise in the prothrombin time with no change in the anticoagulant dosage

Brambel I have a possible explanation for the increased resistance to anticoagulants following K_1 administration Vitamin K_1 having a long hydrocarbon chain (phytyl group) in the number three position remains in the blood stream a much longer period of time whereas the menadione derivatives having smaller mole

with a Kahn pipette and discarded The lower aqueous layer is transferred to a pyrex tube 15 x 125 mm and centrifuged to dislodge droplets of toluene in suspension Any residual toluene is removed as far as possible and an aliquot of the aqueous solution transferred for measurement

Measurement of Phenylindandione Phenylindandione shows a moderate light absorption band at 460 mμ and a very marked band at 338 mμ We have used the absorption band at 338 mμ for estimation of danilone in the extracts since it is least affected by interfering substances Such substances make it impossible to use the 460 absorption band The absorption is measured in a Beckman DU Spectrophotometer using quartz cells Color reactions have been tried such as the Folin Ciocalteau method for phenols but none has been found sufficiently sensitive for the concentrations found in plasma A few preliminary trials suggest that polarographic measurements provide a satisfactory alternative method as regards sensitivity and specificity in place of ultraviolet spectrophotometry In a supporting electrolyte of 0 2 N NaOH a well defined wave is obtained and the diffusion current is directly proportional to the concentration over the range 2×10^{-5}M to 2×10^{-4}M Determinations of phenylindandione were made in aqueous alkaline extracts of plasma without apparent interfering effects

Recovery Phenylindandione added to water and carried through the above extraction procedure is recovered quantitatively When added to plasma approximately 80 per cent is recovered but this is highly reproducible Therefore a standard curve must be prepared with known amounts of phenylindandione added to plasma Other solvents acid concentrations etc did not improve the recovery Normal plasma gives a blank reading corresponding to 0 075 mg per cent in plasma An investigation of the transmission curve of the substance extracted from plasma by the method after administration of phenylindandione indicated that the substance in plasma was unchanged phenylindandione

Determination of Treburon in Blood*

Extraction Procedure The extraction procedure of Monkhouse and Jaques (20) for heparin in blood is followed Blood in the amount of 1 8 ml is drawn and added to 0 2 ml of 3 8 per cent sodium citrate and centrifuged The plasma is removed 1 ml of 80 per cent phenol is added to it and it is shaken vigorously After being allowed to stand overnight the upper layer is pipetted either directly or if necessary after centrifugation This aqueous layer is then washed with 1 ml of ether saturated with saline The ether is pipetted off and the ether remaining in solution is removed by heating in a water bath at 65 C for one to two minutes

Measurement of Treburon Treburon can be measured by its metachromatic effect on azure A in the same way as heparin The color can be best measured in a Lovibond tintometer as described by Jaques Ricker and Bruce Mitford (21) Alternative methods are to measure the absorption at 500 mμ in the Coleman photometer or the absorption ratio 500 540 mμ in the Beckman spectrophotometer While the absorption band for metachromasia is at 500 mμ it is difficult to obtain reproducible results in the Beckman due to variations in the dye Since at 540 mμ the light

*This work has been supported by a grant in aid from the National Cancer Institute of Canada

Overman It has been our experience that if one to two mg of dicumarol are administered daily to the rat mixed with the food or by stomach tube all the rats die within a period of two to three weeks We have not seen any resistance to dicumarol develop

Jaques I should like to remind the group of the data I reported here two years ago on dicumarol labeled with C^{14} (19) That data seemed to indicate fairly conclusively that dicumarol was fixed in the liver as such and that these problems of variations in the effect of dicumarol and factors bearing on the effectiveness of dicumarol could be related to the time that the dicumarol remained in the liver We have continued investigations along that line accumulating further data and the results so far continue to confirm this interpretation

Dr Brambel raised a question concerning the comparison of menadione and vitamin K_1 as regards rate of excretion or loss from the body We have not yet obtained data on K_1 but we have data on menadione labeled with radioactive carbon It does disappear in both mice and cholecystomised dogs at a surprisingly rapid rate With an intramuscular injection of menadione in mice fifty per cent of it is lost from the body in thirty to thirty five minutes

Some data I particularly want to report are in connection with the new anticoagulants phenylindandione and treburon During the last year Mary Ogilvie J Lowenthal and I developed a chemical method for the determination of phenylindandione in blood and Erica Lepp and I developed a chemical method for the determination of treburon in blood A description of these would seem to be appropriate before I go further

Determination of Phenylindandione in Blood*

Extraction of Plasma The blood is drawn using 1/10th volume of 3 8 per cent sodium citrate as anticoagulant and it is centrifuged immediately one ml of plasma is placed in a glass stoppered test tube (20 mm I D x 145 mm) 3 ml of water added mixed 1 ml 1 N H_2SO_4 added with mixing and then 15 ml toluene added The tubes are stoppered and shaken mechanically for 30 minutes They are then allowed to stand about 5 minutes for separation Occasionally an emulsion is obtained which is broken by centrifuging Ten ml of toluene are taken using a 10 ml all glass syringe with a 3 inch 15 gauge needle care being taken not to disturb the interphase and the toluene is transferred to a second clean glass stoppered test tube Four ml of 3 N NaOH are added and the mixture shaken mechanically for ten to fifteen minutes After having stood to allow the two layers to separate the upper toluene layer is removed

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absorption by azure A is not changed by the presence of heparin (see absorption curves Figure 35) by taking the density ratio variations in dye are cancelled out Figure 33 shows the standard curve for the density ratio 500 540 mμ vs concentration of treburon

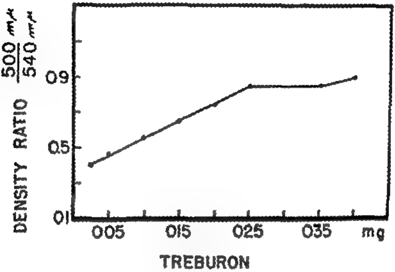

FIGURE 33 Relation of treburon concentration and density ratio 500 540 mμ. Total vol. 4 0 ml 0 1 mg of azure A and M/ borate buffer pH 8 48 Beckman spectro photometer

The thesis was advanced two years ago in my presentation (19) that the differences between dicumarol phenylindandione compound 63 etc could be related to the time that these drugs remain fixed in the liver Judging from the results with dicumarol the blood levels can be used as a means of interpreting the rate of metabolism of the drug I can now report our preliminary studies on phenylindandione in this regard based on blood levels

Ten mg of phenylindandione per kg was administered to rabbits and the blood levels followed As shown in Figure 34 immediately following the intravenous injection the plasma concentration was found to be 12 4 mg per cent A few minutes later it was 6 4 mg per cent The concentration then fell more slowly but none of the drug could be detected in plasma twelve hours after the injection On oral administration a trace of the drug (1 mg per cent) was detected in the plasma This did not appear until three to five hours after ingestion of the drug The first rabbit was fasting the second had been fed prior to receiving the drug Phenylindandione persisted in the blood for four hours only The prothrombin time

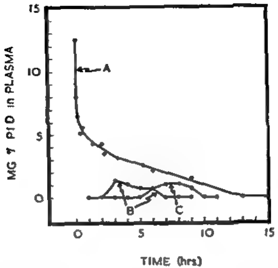

FIGURE 34 Blood levels of phenylindandione when 10 mg/kg was administered to rabbits A intravenous B and C oral administration with B fasting

was not increased until twelve hours after the drug was administered at which time no drug could be detected in the plasma irrespective of route of administration These results are in marked contrast with those obtained by Spinks and I (19) on dicumarol in rabbits Five mg of dicumarol per kg intravenously gave a plasma level of 12 5 mg per cent which fell to 6 mg per cent in one hour Thereafter the plasma concentration fell slowly and the drug did not disappear from the plasma completely until nine days after administration Judging from Shapiro and Weiner s (22) results in man plasma concentrations of dicumarol are very little different with oral or intravenous administration with the exception of the initial rapid fall in concentration (60 per cent of the dose) which occurs in the first two or three hours after intravenous administration

With both drugs upon intravenous administration at this dose level (5 to 10 mg per kg) about 50 per cent of the injected dose rapidly disappears from the circulation The remaining drug then disappears from the circulation following an exponential curve A rough comparison of the two drugs can be made by determining the rate of disappearance of drug from the circulation at the mid point of this curve For the results in Figure 34 a value of 8 3 μg phenyl indandione per ml of blood per hour was obtained while similar

treatment of the data of Jaques and Spinks gives a value of 0 76 µg dicumarol per ml per hour This emphasizes the great difference in rate of metabolism of the two drugs

The effect of repeated administration of phenylindandione has also been studied Eight mg phenylindandione per Kg weight given orally to a dog every eight hours caused the prothrombin time to rise steadily until it reached a level of 75 seconds The plasma drug levels under this regime were comparatively constant (1 2 to 2 0 mg per cent) It is evident from these preliminary results that judged on blood levels phenylindandione is metabolized much more rapidly than is dicumarol

A chemical method for the extraction of heparin in blood was developed by Monkhouse and myself (20) In the present investigation, this method has been successfully applied to treburon As shown in Figure 35 treburon gives a metachromatic color with

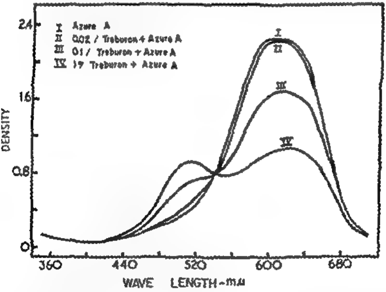

FIGURE 35 Absorption spectrum of azure A and azure A + treburon I azure A II azure A + 0 02% treburon III A + 0 1% treburon IV A + 1 0% treburon

azure A identical to that given by heparin as described by Ricker Bruce Mitford and myself (21) The absorption band of the dye itself at 620 mµ is reduced and a new absorption band at 500 mµ (the metachromatic color) appears

The metachromatic activity of treburon was compared quantitatively with the metachromatic activity of heparin One milligram

of treburon was found to have the metachromatic activity of one milligram of sodium heparin and the substance could be satisfactorily measured quantitatively by using this property Treburon was also assayed by the antithrombin method described for heparin by Charles and myself (23) One milligram of treburon was found to have the antithrombin activity of 1/6 mg of sodium heparin Blood levels of treburon and heparin after intravenous injection in a dog are shown in Figure 36 It can be seen that the blood levels

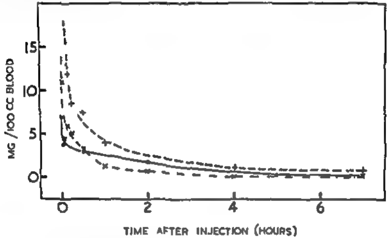

FIGURE 36 Blood levels of treburon and heparin after intravenous injection Dog 22 kg + — — + treburon 40 mg /kg x — — — x treburon 10 mg kg ———— heparin 10 mg /kg

of the two drugs after injection of the same dose (10 mg per kg) are similar and the time for the drug to disappear from the circulation appears to be about the same for the dose of 10 mg per kg Thus it perhaps is necessary to point out is comparing the drugs on a weight basis not on the basis of equal anticoagulant activities

Wright At this time I shall introduce a case history in serial form About four months ago a physician came to my office with a history of bruising easily and bleeding often either spontaneously or after trivial trauma He had been examined by a well known hematologist and a diagnosis of idiopathic hypoprothrombinemia had been made

At New York Hospital he was found to have numerous large hematomas and gross hematuria His story was not quite convincing

and we were suspicious of the possible intake of some anticoagulant although he denied this He did state that a certain type of weed growing in back of his barn was often used in their home salads and ventured the opinion that this might be responsible for his hemorrhagic diathesis At this point I had Dr Overman see him

Overman When this physician came in he had a prothrombin time of approximately 200 seconds We followed him for three days while we were investigating various possibilities One of our procedures included the determination of the dicumarol level of his plasma The plasma contained 56 to 60 gamma on each of the three days we did the determination He was given 500 mg of vitamin K_1 by mouth and within four hours his prothrombin time had fallen to 60 seconds Nevertheless he maintained vigorously that he was not taking dicumarol or tromexan

Wright This presented an embarrassing situation since he was a physician We confronted him with the fact that dicumarol had been found in his blood and said that we were very anxious to continue our studies and work with him He vanished immediately after that conference and that was the last we saw of him However, it was not very long before we heard that he was in the hands of Dr Tocantins

Tocantins This young doctor was referred to us as a possible example of idiopathic hypoprothrombinemia After using plasma and vitamin K this patient s prothrombin which had been very low rose and the bleeding abated Since the nature of the disorder was unknown I was asked to study him and give an opinion

When I first met him I received the same impression that Dr Wright did I had a feeling that there was something unusual about the whole thing particularly since one of his first remarks was that he was having a great deal of pain He asked us to relieve him of that pain and he proceeded to specify exactly what he needed for relief

At the time of admission to the hospital his prothrombin was normal Since we suspected that he was taking a drug — we did not know what it was — in order that he could qualify for another drug we examined his blood four times a day We did assays for pro thrombin and dicumarol in his blood and for dicumarol in the urine which was collected each twenty four hours We had him under observation for about two weeks and during that entire time his prothrombin was normal He must have been amused all this while because he had already been examined by Dr Wright and knew exactly what we were hunting for I asked him one time whether

it would be possible that he was being exposed to a drug of some type He did not think so he said unless "some of these drugs that you are giving me are doing it"

But he was very insistent on wanting relief and even after we told him that his prothrombin had been normal all along he required 400 to 500 mg of demerol daily to control his alleged pain There were no objective physical signs of disease at any time After a two-week interval during which we could not establish that he was taking dicumarol we suggested that he seek the help of one of our psychiatrists But the patient decided to go home and see his own psychiatrist I did not know until today that he was the same man that Dr Wright had seen

Olwin We had a similar case in a nurse from another hospital whom we were asked to see Finally I told the referring doctor that I had never seen the condition except in a patient with dicumarol poisoning He confronted her with that diagnosis but she denied taking dicumarol I suggested that she be confined to a hospital room her clothing searched and all access to drugs denied When he proposed that to her she confessed to taking dicumarol

Wright There are undoubtedly many cases of self medication with dicumarol I thought it would be worth while to include this history in our Transactions because similar patients may be encountered by other physicians who should be alerted to the possibility

Fremont Smith What is the motivation for an individual's taking dicumarol?

Wright With the physician there were probably two factors One was the possibility of being inducted into the Army the second was narcotic addiction It is fair to say however that while the patient was under our care he did not press us for narcotics or even sedatives It was not a major factor then but in the next few months the addiction probably developed

Fremont Smith What was the motivation in the case of the nurse?

Olwin She was a psychiatric case and was looking for attention

Wright Dr Cronkite you have had a case haven't you?

Cronkite Yes she also was a nurse As a matter of fact she was originally studied here in New York by Tagnon * and at that time I believe a diagnosis of idiopathic hypoprothrombinemia was made I saw her accidentally when I was asked to do some studies on her blood Being not too careful about the medical history we com

* Tagnon H J Personal communication Paper in preparation

pletely missed the real diagnosis However she did not have any thing wrong at the time we studied her Later she began to bleed again and was studied elsewhere Vitamin K_1 was given intra muscularly apparently into the region of the sciatic nerve Pain developed, and a generous supply of demerol was given to her *addiction apparently followed* She eventually came back to the Memorial Hospital and was then I believe properly diagnosed Her motivation was not ascertained as far as I know

Wright This suggests that all cases of so called idiopathic hypo prothrombinemia must be carefully reviewed

Brambel Our case was quite interesting The patient had a nor mal prothrombin and then for clinical purposes received large doses of antibiotics penicillin aureomycin terramycin etc Follow ing the antibiotics the patient developed a hypoprothrombinemia which responded only to K_1 and not to the menadione derivatives

When the patient came to us after he had made a tour of the hospitals in Baltimore he just as Dr Tocantins mentioned in his case made a request for demerol In one hospital to which he had been admitted he had been recommended for a psychiatric con sultation This would have been an excellent case — it may still turn out to be — of hypoprothrombinemia secondary to antibiotics but we shall have to be extremely careful in the light of the cases just discussed

Wright Dr Hardin Jones has come from Dr Gofman s group to tell us a little about the relationship of heparin and lipids in clot ting factors

Jones As a spokesman for the Berkeley group * I shall stress the necessity of heparin or heparin like materials for the lipid transport system of the blood This is the core of most of our current thinking

There are a large number of individuals both normals and atherosclerotics *who show deficiencies in the lipid transport system* which seem to be of the nature of metabolic blocks (25 26 27 28 29 30 31 32 33) These metabolic blocks can for a time at least be partly removed as evidenced by the disappearance of the atypical concentrations of lipoproteins of S_f 10 100 and higher by the ad ministration of heparin (24)

In the action of heparin heparin itself apparently is not involved but injected intravenously or otherwise administered to animals

* Gofman John W Lindgren Frank Lyon T P Graham Dean Pierce Frank Nichols Alex Trautman Rodes Freeman Keith Hayes T Hewitt J Biggs M Fritchevsky F Rosenthal D Rubin L

either normal animals or ones having deficiencies of serum lipoprotein metabolism it causes the generation of what we have referred to in our own laboratory as the "active" factor We do not know precisely what the active factor is but it is a substance which seems to initiate some of these conversions of the large lipoprotein molecules from higher S_f to lower S_f

I shall have to go back for a moment and describe the scheme of fat transport as we think it works in the blood (28 29) Beginning from the rather classical concept of lipids coming into the blood stream probably in chylomicrons or just about the size of chylomicrons they are large lipoprotein molecules perhaps even aggregates but it is unnecessary to make a fine distinction Their molecular weight is probably in the upward range about one hundred billion The larger molecules are composed chiefly of neutral fat and in addition contain very small amounts of cholesterol phospholipid and protein (29 34)

Subsequent to the entry of these very large particles into the bloodstream they are chewed up so to speak and redistributed as far as their structural composition is concerned so that the amount of lipid decreases and the amount of protein increases with shifts also in the relative amounts of accompanying neutral fat cholesterol cholesterol ester and phospholipid In this transport system the lipid of the lipoprotein is either passed progressively to newly generated lipoproteins of smaller size greater density and higher protein content or possibly the lipid is progressively removed in small molecular weight fragments leaving a core lipoprotein that becomes progressively higher in density and hence of lower Svedberg flotation (S_f) rates as measured in the ultracentrifuge

The entire system of lipoproteins may be given a general classification in the ultracentrifuge by a measure of the characteristic migration or flotation rates of these various lipoprotein fractions This depends largely upon the amount of lipid material they contain and their molecular size in the ultracentrifuge At least nine discrete lipoproteins have now been characterized in human blood (29) Further work is necessary to determine where these fractions fit into the Cohn scheme of identification (34 35)

The conversion of blood lipids appears to start with the molecules of the chylomicron or near chylomicron size and progressively they are rearranged into molecules that are smaller and that contain more protein relative to the lipid content The chylomicrons and a group of slightly smaller lipoproteins ranging from chylomicrons (S_f 40 000) to the S_f 100 class are associated with alimentary lipemia

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and normally constitute under 100 milligrams per cent of the serum lipids As the period past fat ingestion increases the predominant alimentary lipoprotein approaches S_f 100 presumably by the con version of higher S_f material to S_f 100 and lower classes

In the true normal there are probably only traces of lipoprotein in the classes between S_f 100 to S_f 6 The dominant lipoprotein normally present in the blood is in the S_f [illegible] range It is 30 per cent cholesterol and usually accounts for the bulk of cholesterol asso ciated with blood lipids (28 29 34) Between the various classes of lipoproteins there are only weakly established relationships and this is true at any level of serum cholesterol The amounts of any class of lipoprotein cannot be predicted with certainty from the con centration of any other class (28 29)

In individuals who have accumulations of serum lipoproteins in the S_f 40 000 100 greater than 100 mg per cent S_f 100 70 S_f 70 S_f 20 S_f 20 10 and occasionally a hyperlipemia of S_f 6 or a com bination of these classes we believe there essentially are metabolic blocks against normal conversion rates of these serum lipids along the lipoprotein chains, and hence a piling up of the lipoproteins occurs at the slowest steps of the conversion process Why there should be several variations of positions of these blocks with their associated serum lipoprotein patterns is unknown

The giving of heparin parenterally has uniformly generated a lipoprotein-converting active factor and the "active factor or factors progressively convert lipoproteins of high S_f to lower S_f *In vivo* this occurs at an obviously accelerated rate on the higher S_f molecules (24) All classes of lipoproteins have been observed to be influenced by heparin administration eventually

The uniform action of heparin in converting high S_f lipoproteins to lower S_f lipoproteins follows a pattern remarkably similar to the conversion of a single load of high S_f serum lipids when such a lipoprotein fraction is isolated and injected intravenously as a momentary load in the rabbit* (28 29)

Tocantins Is there any difference in the size of the lipoprotein molecules in blood entering the liver and those leaving the liver?

Jones We have not done studies on the portal blood as compared to the hepatic blood But we are beginning to do some on thoracic lymph There the molecules are the size of chylomicrons or just below that size in moderate amount What the whole lipoprotein structure of lymph is I do not know

* Pierce F T *Serial interconversions of serum lipoproteins in vivo* (To be pub lished)

Much of the thinking on this problem will be helped by actually finding out what the active factor is and what role the active factor may have if any in the control of the coagulation process Some of the fractions that have been isolated have lipoprotein conversion action and they seem to be identified with materials corresponding in density with proteins that have some lipid attachments There is some suggestion at the moment that certain of these active factors may be lipoproteins but that other active factor substances may be essentially free of lipid material So we are dealing with a reaction of a possibly complex character all being materials of a similar action generated by the administration of heparin

Neither is it known whether the active factor itself contains heparin It may or may not We have no way of knowing until we isolate the material for chemical analysis

It is evident that the large group of people who are candidates for anticoagulant therapy are mainly those that by this theory have heparin like deficiency in their fat metabolism Since this is so it might be preferable to consider some of the aspects of the physiology of heparin in this whole process as compared to empirical management of such patients with general anticoagulants

As far as we know from moderate study of serum from patients before and after dicumarolization there is no effect on lipoprotein metabolism induced by the dicumarol series On the other hand a study of many heparin like materials shows that they have in general a similar action and that they can all generate the substances that cause serial lipoprotein transformation Paritol C (Wyeth) has been studied Also a sketchy amount of information is available on treburon which seems to possess the same conversion property as do other materials of the sulfonated polysaccharide type But since we are trying to unravel the physiological mechanisms here involved we prefer to keep most of the work on the basis of investigation of heparin itself because it is at least a physiological substance with very low toxicity

It has been previously reported by the clinical workers associated with this that heparin does produce beneficial results in the relief of pain associated with coronary insufficiency Whether this action of heparin is achieved through its influence on the lipid transport mechanism we do not know The total quantity of heparin used in these cases namely about 100 mg parenterally in frequencies ranging from once a day to once a week is insufficient in terms of the amount of heparin that would be required to bring about normal lipid transport for more than a few hours at a time Two

patients with xanthoma tuberosum have been given heparin in the range of 100 mg per day for six months Their S_f 10 100 lipoproteins have been reduced from over 1000 mg per cent to about 300 mg per cent as the maximum level between injections of heparin

The amount of heparin that is converted into the active factor is variable and it is probably only a very small quantity of the heparin administered at any one time For this reason we have thought in terms of giving subcutaneous heparin in a form that might release the heparin slowly over a period of time which should result in a greater generation of the lipoprotein converting material

Tocantins You speak of heparin being converted into the active factor *Is that actually a conversion of the heparin?*

Jones I mentioned a moment ago that this could be a conversion of heparin or a release of the substance by heparin We do not know which of these two is correct

Tocantins Because it is not converted *in vitro*

Jones No it is not converted *in vitro* and heparin will not generate the material *in vitro* in blood or serum

Allen Could it be a combination of heparin with something else?

Jones Yes it must be a combination with some other material

Tocantins That would be an active factor deficiency not an acute heparin deficiency

Jones Whichever way you want to look at it At least the heparin like mechanism is deficient

Best I wonder whether Dr Jones means fat transport or just the state of the fat in the blood?

Jones It is the state of the fat in the blood certainly but there must be consideration of the amount of fat there and the turnover of the amount of fat present in these blood lipoproteins

Best I have heard no evidence from you nor seen evidence in the journals that there is any change in fat transport at all under any of the conditions you have described *I know of no proof that* heparin causes any change in fat transport and it is a field in which I have worked a great deal

If insulin is taken away one finds 40 per cent fat in a liver that had none Take choline away and there is a terrifically fatty liver And I think we demonstrated that with the pituitary factor fat can be transported

I have worked on heparin too and have seen no evidence that it causes a change in the transport of fat We did not have an ultracentrifuge so we will accept your data on the state of fat in the

blood But I have yet to find any evidence in your publications or statements that heparin affects the transport of fat

Jones We have generally referred to these blood lipids as lipids in the state of transport and we have described serial lipoprotein conversion in the blood with lipid entering and going somewhere At least they are being moved around in the blood

Best How do you know they are? They may be just staying there

Jones They may be just staying there but they are being renewed without reference to any concentration in the blood

Best When you talk about transport you have to prove some shift I think

Jones Fat which in man may amount to about 100 to 200 Gm per day may be introduced into the very large molecular weight fraction of the blood and somehow or other the fat is reorganized into these smaller molecular weight lipoprotein molecules in the blood before the lipid leaves the blood This is what we have referred to as the blood lipid transport system

Best I think your evidence completely concerns the state of fat in the blood

Alexander The state of the *circulating* fat

Warner Does that progressive change in the physical state of the fat in the blood occur in a sample that is withdrawn?

Jones A sample will stay at a static point representing the pattern conversion of lipoprotein at the time the blood was drawn

Warner So this all has to be done *in vivo*

Jones The lipids appear to go down in sequence starting with the chylomicrons and going through a chain that may involve serially all of the plasma lipoproteins

Best The heparin works *in vitro* surely changing the state of the fat This is perhaps controversial?

Jones No drawn blood mixed with heparin will show no changes in lipoprotein type or concentration as we have studied lipoproteins

Tocantins I wonder how much of the separation or splitting of the fat as it might be called is due simply to the force of the circulation in jamming blood through very finely divided capillaries? If a thick emulsion of fat made up of quite large globules is put in a Logeman homogenizer (the working principle of this homogenizer being that of pushing a column of liquid under high pressure through a very fine aperture and jamming the liquid against a flat surface on the other side) an almost transparent solution and a splitting of the globules of fat is obtained Is it possible that in the circulation a similar process is taking place so that the large vessels bring these

large fat particles to the capillary bed where they are subdivided and turned into a fine suspension?

Jones Although very large chylomicrons or free fat droplets might be so affected, I completely doubt that such a small force could be a factor altering serum lipoproteins Also, a person who has an accumulation of abnormal material has his blood in a state of movement through the capillary bed and just the addition of heparin will cause immediate shifts in these molecules Also if some of these S_f 10 100 lipoproteins are mixed with the active factor that is present in plasma or serum withdrawn after heparinization some of these shifts can be produced *in vitro*

Alexander What is the evidence for an active factor? You use the words active factor but could it be a deficiency or could it be a physicochemical difference? Do you have evidence that if you give the person who has this abnormal pattern of circulating fat a transfusion from a normal individual it will be rectified? In other words what is the evidence that you have a "phenomenin * and not a phenomenon?

Jones We are not able to be certain about the presence of this active material in the blood of normal people Let s take some people that do not have these metabolic disturbances of serum lipids We can suppose that they possess at least a sufficient amount of this lipid converting material

Alexander Or a different type of abnormality not necessarily a factor

Jones You can presume anything But to continue this thought we have not been able to find such active materials at detectable levels in normal blood In the case of some individuals it seems to be suggested that their serum mixed with serum containing these abnormal molecules will cause *in vitro* changes similar to those of the active factor But this is very preliminary and we could not say that such a person has an active factor in his blood

Tocantins But you can bring the active factor about in every individual by injecting heparin?

---

* EDITOR S NOTE In answer to a letter asking for a definition of *phenomenin* Dr Alexander replied as follows I used the word *phenomenin* somewhat jocularly contrasting it with the word *phenomenon* The late Professor Lawrence J Henderson was very critical of physiologists and biologists who were all too ready to attribute an observed phenomenon to a substance before the entity was clearly established to exist He used to say "The trouble with many workers is that they attribute certain observations to a *phenomenin* (a substance) rather than to a *phenomenon* before they have separated the substance and shown that it actually exists

Jones Each time it has been looked for we have seen it in every individual who has received heparin

Mann I think it should be brought out that in 1943 Dr Paul F Hahn (36) reported that intravenous heparin abolished alimentary lipemia Like Dr Best Hahn did not have an ultracentrifuge He made the simple visual observation that the turbidity of plasma due to alimentary lipemia disappeared very rapidly *in vivo* following the injection of heparin Much less heparin was required to clear alimentary lipemia than to affect coagulation appreciably

Heparin added to lipemic plasma *in vitro* does not cause clearing Dr Block Dr Barker and I (37) have found that the minimal dose of heparin which causes clearing of alimentary lipemia in normal persons frequently fails to clear alimentary lipemia in atherosclerotic individuals This resistance to the clearing of alimentary lipemia by heparin does not appear in all cases of atherosclerosis however

Allen Can you increase that dose and get a clearing?

Mann Oh yes with a large dose every one clears

Flynn Didn t Anderson and Fawcett (38) show that the reaction could be started *in vivo*?

Mann If it is started *in vivo* it will then continue *in vitro*

Warner Will the plasma of a heparinized individual clear the lipemia in another plasma?

Mann Yes *in vitro* That is what Anderson and Fawcett found Weld (39) showed that this peculiar reaction did not require a specific organ but would occur in any organ We have confirmed this in demonstrating the reaction in hepatectomized dogs and in a preparation with the aorta ligated at the diaphragm The one thing Weld reported that we have not been able to confirm is the production of clearing in whole blood *in vitro* I believe that others have also been unable to do this

Jaques I should like to ask Dr Jones how he is able to talk about a heparin deficiency in relation to this particular situation

Jones It is entirely a hypothesis The best story that we can put together at this point is that the basic deficiencies that we are seeing may represent a heparin deficiency We do not claim that we have proved that they represent a heparin deficiency In all cases where we see metabolic blockages in the conversion of these molecules we can cause the disappearance of the atypical molecules by giving heparin which induces a reaction that seems to follow the normal manner of lipoprotein conversions

Best Have you confirmed your finding that heparin prevents atherosclerosis in cholesterol fed rabbits?

Jones Yes (24) I am not prepared to quote numbers because the data are just being gathered now and are in a preliminary state but heparin will retard the development of atherosclerosis in rabbits that are similar in every way except for their receiving 20 mg of heparin per day to control cholesterol fed rabbits that are developing atherosclerosis *

Best Dr J B Firstbrook in my laboratory is doing an experiment in which cholesterol fed rabbits are receiving heparin The experiment is not finished but one or two of the rabbits receiving heparin have died and they had full blown atherosclerosis

Jones I am sure that many of the rabbits will have atherosclerosis The observation we have made is that heparin injected rabbits have less atherosclerosis than the cholesterol fed controls Under circumstances in which heparin is given once a day there would only be a fraction of the day in which the S_f 10 100 lipoproteins would be suppressed and at twenty four hours after heparin there may be no difference between serum lipids of either heparin injected or control rabbits

Best I take it that this is a test which people without an ultra centrifuge can carry out so you will very likely be challenged or confirmed before very long

Wright It hardly seems necessary to remind this group that there are several substances which prevent atherosclerosis in cholesterol fed rabbits but which have no effect on atherosclerosis in man I think we must be very cautious about drawing any conclusions regarding the effect of heparin in man

I might also mention the fact that the angina syndrome has been reported as improved by everything from testosterone to dicumarol and now we have heparin Angina is subjective and it is not possible to analyze it quantitatively It is one of the most difficult signs or symptoms for a clinician to evaluate We were able to produce a high percentage of relief in controls with placebos when we were first trying testosterone for anginal pain It is much more difficult to evaluate than intermittent claudication produced on a treadmill run at a consistent rate Dr Eugene Simon of our group is presently constructing a new treadmill to test heparin and determine whether

* We now realize that a somewhat wider range of cholesterol dosage is needed to produce a given degree of atherosclerosis in a given stock of rabbits Also the amount of heparin or the heparin cholesterol ratio is a factor which must be arrived at anew in each laboratory area dependent upon the rabbit stock If an excessive amount of cholesterol is fed relative to the atherogenesity of the stock even 20 mg of heparin per day may be insufficient to give significant improvement of serum lipoprotein pattern and protection from atherosclerosis

we can obtain any quantitative effect I think Nelson Barker and others who work with peripheral vascular disease will testify that that is a very difficult thing to do But with a treadmill that can be adjusted to different speeds I think we may be able to obtain some evaluation provided that the differences are great enough Dr Gofman has told me that others have raised the same question It should be in our minutes that the question is seriously raised that we recognize that this symptom is extremely difficult to evaluate This is said in sympathy toward any group that tries to evaluate it because I know how difficult it will be

Jones It is impossible to present all of the side observations which have been a part of our studies of the whole problem For example in cases of acute myocardial infarction there is a significant tendency for those having high S_f 12 20 levels after infarction not to survive (28) Conversely a person with low S_f 12 20 levels has a better chance for recovery Such individuals tend to have even lower S_f 12 20 levels than they had when studied earlier or when they were subsequently studied after the acute period There is also some evidence that the acute infarctives have been able to generate detectable levels of the lipoprotein converting active factor *

These are some of the things that are a part of the whole each needs further investigation

Certainly all of the observations made so far can be related to the original observation of Hahn (36) which we have credited and which we feel is a basic observation on the role of heparin

* Rubin L and Nichols A Unpublished observations

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this first reaction could not be elucidated since prothrombin also entered the second reaction which was proceeding simultaneously Various findings however indicated that a substance other than prothrombin but present in the prothrombin preparations was active in the formation of the prothrombin converting principle factor VI This unknown factor was provisionally named *cofactor V*

The existence of cofactor V was indicated by the following findings Prothrombin prepared in different ways showed great variation in the ability to form factor VI crude preparations being more potent than partly purified preparations It was also found that by applying constant concentrations of proaccelerin (factor V) thromboplastin and calcium the rate of thrombin formation varied with the degree of purity of the prothrombin preparations used These findings were suggestive of the presence of various amounts of a converting factor differing from factor V in the prothrombin preparations used Experiments designed to isolate this new converting factor cofactor V from human plasma failed at that time however and no further progress was made on this problem until 1949

In the meantime Seegers and his colleagues (3) demonstrated that plasma Ac globulin which is identical with proaccelerin is activated to serum Ac globulin by an effect of thrombin I confirmed this finding By the addition of a small amount of thrombin to plasma or to a preparation of proaccelerin the effect of these substances on shortening the clotting time of plasma from a parahemophilic was greatly increased Proaccelerin activated in this way has been termed accelerin

The activation of proaccelerin by thrombin also explained why prothrombin had to be present for the production of factor VI It had been demonstrated previously however that the addition of thrombin to a mixture of proaccelerin thromboplastin and calcium did not produce any factor VI activity detectable by the method used (1) Accelerin or serum Ac globulin therefore was not identical with factor VI Accelerin (produced by adding thrombin to proaccelerin) when thromboplastin and calcium were added still needed the presence of a prothrombin preparation to give formation of factor VI Normal serum therefore contains a factor in addition to accelerin probably produced by the interaction of thromboplastin and cofactor V to constitute the complete prothrombin converting principle named factor VI This concept is illustrated by the formula in Figure 38

NEW CLOTTING FACTORS

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I SHALL LIMIT my remarks to my own investigations regarding the new factors proaccelerin accelerin proconvertin and convertin * In 1943 I described a disease that I called parahemophilia I demonstrated that this disease was caused by the lack of a previously unrecognized clotting factor the fifth clotting factor or factor V Later I renamed this factor proaccelerin a name proposed by Astrup in Denmark From 1943 to 1945 further investigations showed that thrombin formation occurred as follows (1 2)

$$\left.\begin{matrix}\text{Cofactor V}\\ \text{Prothrombin}\end{matrix}\right\} + \text{Factor V} + \text{Thromboplastin} + \text{Ca} \rightarrow \text{Factor VI}$$

$$\text{Prothrombin} \xrightarrow{\text{Factor VI} + \text{Ca}} \text{Thrombin}$$

FIGURE 37 The theory of thrombin formation 1947 Reprinted by permission from Owren P A Coagulation of blood investigations on new clotting factor *Acta med scandinav* Supp 194 1 (1947) and from Owren P A New factors concerned in coagulation of blood *Bull schweiz Akad d med Wissensch* 3 163 (1947)

In these investigations it was observed that during the formation of thrombin there occurs a new principle provisionally named factor VI which arises *de novo* and is the active principle in converting prothrombin to thrombin in the presence of calcium It was further demonstrated that factor V has a decisive influence on the maximal activity of the factor VI formed indicating that factor V is integrated with factor VI wholly or partly It was also shown that thromboplastin and calcium alone did not react with proaccelerin to form factor VI and this reaction needed the presence of a prothrombin preparation The role played by prothrombin in

* EDITOR S NOTE See appendix for the synonymy of these terms

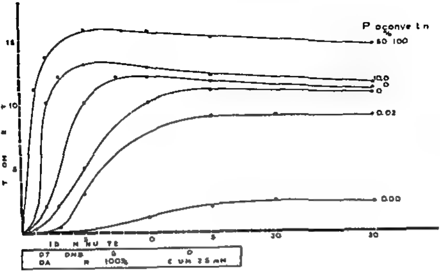

FIGURE 39 The effect of proconvertin concentration on the thrombin formation

$$\text{Proconvertin} \xrightarrow{\text{Thromboplastin + Calcium}} \text{Convertin}$$

$$\text{Prothrombin} \xrightarrow{\text{Convertin + Accelerin + Ca}} \text{Thrombin}$$

Parahemophilic Plasma + Ca	Without Accelerin	10 Minutes
	Incubation Time Before Addition of Accelerin	Clotting Time
Addition of Accelerin	0 sec	69 sec
	30	39
	60	30
	90	27
	120	25

Parahemophilic Plasma + Thromboplastin Dil 1 50 + Ca	Without Accelerin	120 Sec
	Incubation Time Before Addition of Accelerin	Clotting Time
Addition of Accelerin	0 sec	28 sec
	15	16
	30	11 4
	45	11 2

FIGURE 40 The appearance and activity of convertin during incubation of procon vertin thromboplastin and calcium tested by the addition of accelerin

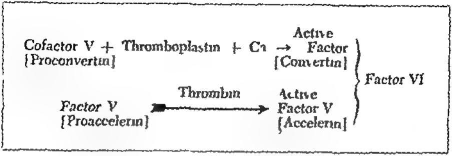

FIGURE 38. The formation of factor VI

The presence of such an additional active factor in serum was also confirmed by the following findings In contrast to fresh normal serum which always showed a high factor VI activity the serum from plasma defibrinated by thrombin showed no factor VI activity in spite of both serums containing accelerin When tested for accelerin activity (applying the proaccelerin method using parahemophilic plasma as a reagent and using 100 units per ml as the normal proaccelerin concentration in human plasma) it was found that serum obtained by the defibrination of plasma by thrombin showed an activity corresponding to 500 units per ml of serum Normal serum after spontaneous clotting often showed an activity of 600 or 700 units and after addition of thromboplastin the activity increased to 5000 units per ml Serum especially after the addition of thromboplastin, therefore contained a substance potentiating the effect of accelerin on the proaccelerin free plasma

It was not until 1949 (4) however that we succeeded in the fractionation of a prothrombin preparation into two factors one being a converting factor cofactor V and the other being prothrombin itself The name of cofactor V was later changed to *proconvertin*

The effect of proconvertin thus prepared on the conversion of purified prothrombin is illustrated in Figure 39 When thromboplastin calcium, and proaccelerin are present in constant amounts the velocity of thrombin formation rises with increasing concentrations of proconvertin up to a certain limit Without the addition of proconvertin the thrombin formation takes place very slowly

The existence of convertin was confirmed by experiments on plasma from a parahemophilic where there was no disturbance caused by the proaccelerin accelerin system (5)

Plasma from a parahemophilic patient gave a unique opportunity to follow the formation of convertin because accelerin could be

from plasma we observed that one of these factors was removed by passing oxalated ox plasma once through filter pads containing 20 or 30 per cent asbestos By repeated filtrations through filter pads containing 30 per cent asbestos or more both factors were removed The filtrate after 20 per cent asbestos was used showed a prolonged clotting time by recalcification both with and without added thromboplastin The addition of normal serum however restored the clotting time to normal The active factor in serum was stable on storage disappearing very slowly in the course of days and weeks In our first reports this factor was mistaken for prothrombin itself and the factor remaining in the filtrate was taken as proconvertin (4 5) This interchange of prothrombin and proconvertin was caused by the fact that prothrombin at first was not discovered in the filtered plasma Subsequent experiments with the factors isolated from the filtered ox plasma and from serum showed that the serum factor was identical with the proconvertin previously isolated by fractionation of a crude prothrombin derived from ox plasma The filtered ox plasma was free of proconvertin but contained prothrombin (6) Seegers* first suggested that the serum factor was proconvertin and Koller (7) using my technique came to the same conclusion independently

The selective adsorption of proconvertin from oxalated ox plasma by 20 per cent asbestos filter pads provides a reagent for the quantitative estimation of proconvertin (8) About 60 to 70 per cent of

	CONSTANT	REAGENTS
Determination of Proconvertin	Thromboplastin	Human Brain Extract
	Calcium	Calcium Chloride
	Proaccelerin	Proconvertin free Ox Plasma
	Prothrombin Fibrinogen	Prepared by passing through Asbestos Paper Filter Pads 20%

	CONSTANT	REAGENTS
Determination of Proaccelerin	Thromboplastin	Human Brain Extract
	Calcium	
	Prothrombin Proconvertin Fibrinogen	Proaccelerin free Plasma [from Parahemophilia]

FIGURE 47 Principle and reagents for determination of proconvertin and proaccelerin

* Personal communication November 1950

added at any time that we wished to test for the unknown convertin concentration (Figure 40) Recalcified oxalated plasma from this patient showed a clotting time of 10 minutes Addition of a potent accelerin simultaneously with the calcium reduced the clotting time to 69 seconds The plasma before the addition of accelerin was then incubated with calcium in order to see if calcium, together with the patient s own thromboplastin of plasma origin does cause the formation of convertin Figure 40 shows that this does happen The clotting time decreases with increasing incubation time showing that a substance with increasing activity occurs during incubation which gives thrombin formation in the presence of accelerin With the very long clotting time in this plasma we need not consider that there is any thrombin formation in the short incubation time

By adding thromboplastin along with the calcium the clotting time *is 120 seconds During incubation the convertin concentration* rises rapidly as shown by the decrease in clotting time from 28 seconds to 11 2 seconds

These investigations led to the view regarding the position of proconvertin in the events of blood clotting shown in Figure 41

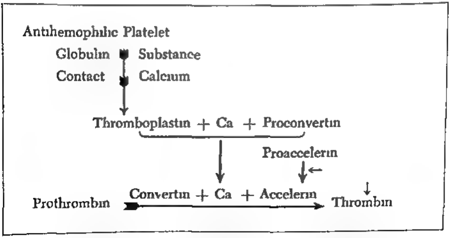

FIGURE 41 Position of proconvertin in blood coagulation

It follows that convertin and accelerin together constitute the prothrombin converting principle previously named factor VI or prothrombinase

During experiments performed in order to obtain a separation of prothrombin and proconvertin by selective adsorption direct

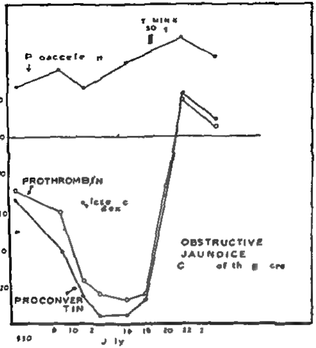

FIGURE 44. The behavior of proconvertin in K avitaminosis (obstructive jaundice)

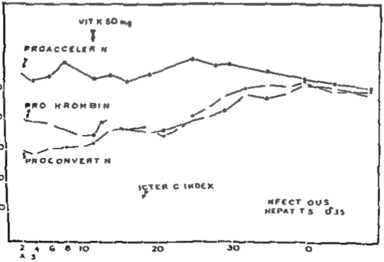

FIGURE 45. Proconvertin and prothrombin in hepatitis

the original prothrombin content in the ox plasma remains in the filtrate Figure 42 shows the principle and reagents for this method

For the quantitative determination of proaccelerin we have used a plasma free of proaccelerin as a reagent usually the plasma from a patient with parahemophilia Artificially prepared proaccelerin free plasma obtained by storage of oxalated human plasma may also be used but the results are more erratic because the storage changes the reactivity of the fibrinogen

By filtering oxalated ox plasma through filter pads containing 40 or 50 per cent asbestos both prothrombin and proconvertin are removed This plasma contains fibrinogen and proaccelerin the latter in three or four times the concentration present in human plasma By adding thromboplastin and calcium to such a filtrate we have a clotting mixture which reacts to the addition of prothrombin and proconvertin This method which gives a quantitative determination of the combined effect of a simultaneous reduction of prothrombin and proconvertin we have named the P & P method (8) It has been used to follow the action of dicumarol because dicumarol causes reduction both of prothrombin and proconvertin By adding a high and constant amount of proconvertin to this reagent it provides a reagent for the specific determination of prothrombin as illustrated in Figure 43 These methods have been described in detail previously (8) (This and the following investigations were done in collaboration with Knut Aas)

	CONSTANT	REAGENTS
Determination of Prothrombin	Thromboplastin	– Human Brain Extract
	Calcium	
	Proconvertin	– Human Serum
	Proaccelerin, Fibrinogen	– Adsorbed Ox Plasma

FIGURE 43 Principle and reagents for the specific determination of prothrombin

Proconvertin decreases simultaneously with prothrombin during treatment with dicumarol and phenylindandione but often to a larger extent than prothrombin in the initial period of treatment (58) The P & P method therefore has proved especially safe and valuable for controlling dicumarol therapy

Proconvertin was also found to decrease roughly parallel to prothrombin in K avitaminosis (Figure 44) and in parenchymatous liver disease (Figure 45) findings which indicate that proconvertin

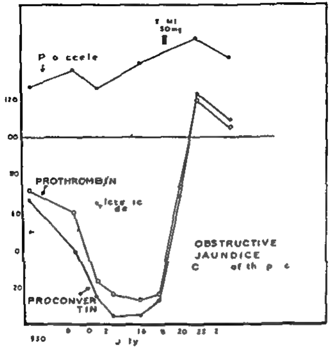

FIGURE 44 The behavior of proconvertin in K avitaminosis (obstructive jaundice)

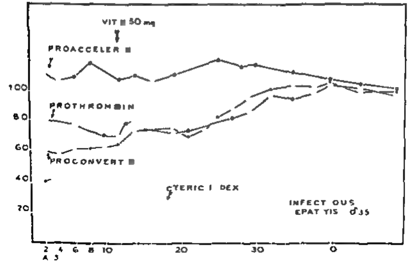

FIGURE 45 Proconvertin and prothrombin in hepatitis

like prothrombin, is synthesized in the liver and that this process needs the presence of vitamin K. This new factor, proconvertin, therefore, in many respects shows a similar behavior to that of prothrombin.

By applying the proconvertin method for the testing of serum during and after coagulation, very high activities were found. This high activity probably was produced by the combined effect of convertin, accelerin, and the residual nonactivated proconvertin. The method did not allow for any differentiation between these activities. By decalcification of the serum at various intervals followed by storing, we found, however, that the activity recorded by the proconvertin method decreased in a few days to a constant low level which then remained unaltered for days and even weeks. This remaining activity was assumed to be caused by residual proconvertin because both accelerin and convertin were found to be unstable on storage. The finding indicated that only a part of the proconvertin present in plasma is activated during the normal clotting process. The residual proconvertin activity in normal serum determined in this way was found to be between 70 and 90 per cent (Figure 46). Accelerin, which is formed from proaccelerin,

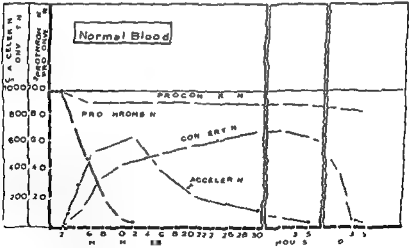

FIGURE 46. Consumption of prothrombin and proconvertin and formation of convertin and accelerin during and after clotting of normal blood.

disappears in some hours or a day. Convertin, which is formed from proconvertin and thromboplastin, disappears in four or five days. Proconvertin then remains as the only residual clotting factor and

is stable for weeks Stored serum therefore is the best source for the preparation of isolated or concentrated proconvertin

Link Is that human serum?

Owren Yes

Last year (1951) a 38 year old man was admitted to our department for a hemorrhagic diathesis present since early childhood There were no cases of a bleeding tendency in his family From the age of three he had suffered from frequent nosebleeds bruises and so forth and upon one occasion he had had a traumatic hemorrhage into his right knee joint The clinical picture was much the same as that seen in parahemophilia the bleeding tendency being less severe than in hemophilia His clotting time was only slightly prolonged 14 minutes against an upper limit of 10 5 minutes (mean time 7 5 minutes) for the method used Quick s clotting test showed 55 to 60 seconds (normal 14 seconds) while prothrombin determined by our specific prothrombin method was 95 per cent of normal Proaccelerin was determined to 98 per cent but estimation of proconvertin by the method mentioned showed only 3 per cent The bleeding time platelet count and also the capillary fragility were all normal Fibrinogen 0 34 per cent ascorbic acid 0 10 mg per 100 ml calcium 9 6 mg per 100 ml

Experiments on the addition to the patient s plasma of small amounts of various plasmas as shown in Figure 47 confirmed our

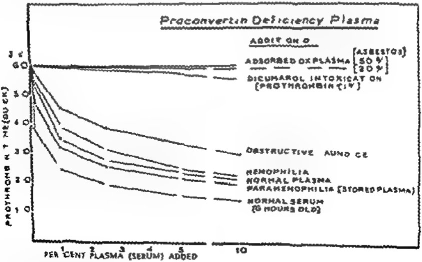

Figure 47 The effect of different plasmas on the prothrombin time of plasma from a case of hypoproconvertinemia

previous findings concerning the behavior of proconvertin. It follows that proconvertin is lacking in ox plasma which is filtered through asbestos filter pads of 20 per cent concentration or higher. It has almost completely disappeared in severe dicumarol intoxication, it is reduced in obstructive jaundice but it is normal in hemophilia and in parahemophilia. The activity of serum is higher than that of normal plasma due to the fact that serum also contains convertin and accelerin.

Oxalated or citrated plasma from this patient provided an excellent reagent for the quantitative determination of proconvertin. This reagent was superior to the proconvertin free ox plasma used previously because of its much higher concentration of prothrombin.

The method I outlined for the determination of proconvertin cannot be applied to fresh serum because the presence of convertin and accelerin will shorten the clotting time found by this method. Neither can it be applied as a convertin method owing to the accelerin present in serum. Similarly the proaccelerin method using proaccelerin free plasma or plasma from a parahemophilic as a reagent cannot be used for serum neither as a proaccelerin method nor as a method for accelerin determination. The presence of convertin shortens the clotting time thereby producing falsely high values.

Based on the final and main reaction in the thrombin formation, the conversion of prothrombin by convertin and accelerin, methods have now been worked out for the specific determination of convertin and accelerin also. These methods may be applied in mixtures containing both these components or their precursors simultaneously. *Principles and reagents are illustrated in Figures 48 and 49.* For the determination of convertin the reagent used contains an excess of accelerin and vice versa.

Accelerin may be prepared by the activation of proaccelerin in adsorbed ox plasma by thrombin. The other main reagent for the convertin method is a proconvertin free plasma. Thromboplastin ■

	CONSTANT	REAGENTS
Determination of Convertin	Prothrombin	Proconvertin free plasma
	Accelerin	{ Adsorbed ox plasma { Defibrinated by thrombin
	Calcium	Calcium Chloride

Figure 48. Principle and reagents for convertin estimation.

	CONSTANT	REAGENTS
	Prothrombin	Proaccelerin free plasma
Determination of Accelerin	Convertin	{Human serum stored and }then Thromboplastin added
	Calcium	Calcium Chloride

FIGURE 49 Principle and reagents for convertin estimation

not added in any of these methods The clotting mixture containing prothrombin and accelerin as reagents reacts specifically with the amount of convertin added The practical procedure is in other respects similar to other one stage methods (8) The presence of proconvertin in the solution to be tested has no or minimal influence on this system because of the very low concentration of thromboplastin present Any influence from small amounts of accelerin in the solution to be tested is eliminated by the high accelerin concentration in the reagent used

The specific determination of accelerin follows a similar principle (Figure 49) The convertin reagent used in this method may be obtained from stored accelerin free serum by activating the residual proconvertin with added thromboplastin

If thromboplastin is added as a reagent in these methods the convertin method will be changed to one also reacting with proconvertin and the accelerin method to one reacting with both proaccelerin and accelerin

The same principle may be used in a new one stage method for the quantitative estimation of prothrombin as illustrated in Figure 50

	CONSTANT	REAGENTS
Determination of Prothrombin	Accelerin	{Adsorbed ox plasma }defibrinated by Thrombin
	Convertin	{Human serum stored and }then Thromboplastin added
	Calcium	Calcium Chloride

FIGURE 50 Principle and reagents for prothrombin estimation

From a theoretical point of view all these methods have the advantage of being based on a single reaction while other one stage methods record the total time for different reactions includ

ing among others the formation of convertin and the activation of proaccelerin to accelerin reactions which to a large extent overlap each other From a practical point of view they have one drawback the fact that convertin and accelerin have hitherto been difficult to obtain in a stable form A constant reactivity of these systems is difficult to assure therefore when the reagents must be kept in solutions in the laboratory for many hours

As a consequence of the new findings we have also modified our two stage prothrombin method introducing convertin and accelerin as converting agents instead of the thromboplastin and proaccelerin previously used

Figure 46 illustrates some results obtained by using these methods for following the formation of convertin and accelerin during spontaneous clotting of normal blood Convertin is formed slowly and reaches a maximum in about half an hour The rate of formation and maximum of activity depends on the thromboplastin present The accelerin formation proceeds more rapidly

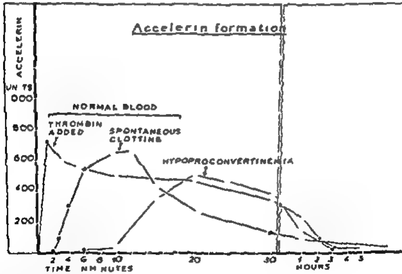

FIGURE 51 The formation of accelerin during spontaneous coagulation and by thrombin defibrination

As shown in Figure 51 the accelerin activity in serum after spontaneous coagulation of normal blood is about 700 units estimated by the specific accelerin method This activity is the same as the activity found in serum obtained from the same plasma by

the addition of thrombin The activity of accelerin in normal serum is not increased by the addition of thromboplastin Serum from the case of hypoproconvertinemia shows about the same activity of accelerin as normal serum The accelerin formation therefore is not directly related to thromboplastin or proconvertin These findings confirm the opinion that proaccelerin is activated to accelerin by thrombin as first pointed out by Ware and Seegers

On the other hand it can be demonstrated that the formation of convertin bears no relation to the presence or absence of proaccelerin As shown in Figure 52 congenital hypoproaccelerinemia

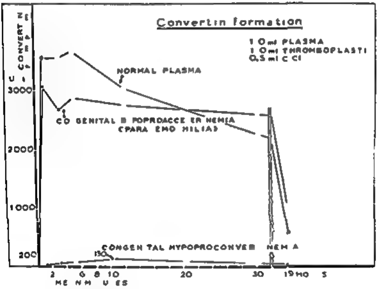

FIGURE 52 The formation of convertin in various plasmas with added thromboplastin

shows the same maximal concentration of convertin with the addition of thromboplastin as does normal plasma The same holds true also for hemophilia with the addition of thromboplastin In congenital hypoproconvertinemia however the maximal convertin production after thromboplastin is added is very low 130 units This finding confirms the fact that proconvertin is necessary for the formation of convertin The convertin concentration found in this case corresponds to a proconvertin concentration of 2 6 per cent which is practically the same as is found by the direct estimation when proconvertin free ox plasma is used as a reagent

To go on to a discussion of the formation of convertin and its quantitative relation to proconvertin and thromboplastin Figure 53

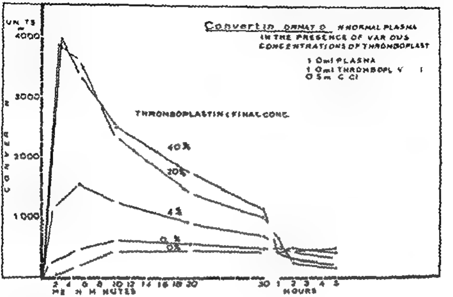

FIGURE 53 The relation between convertin formation and the concentration of thromboplastin

shows that by a constant amount of plasma i e by a constant proconvertin concentration and varying thromboplastin concentration the amount of convertin formed rises with increasing amounts of thromboplastin up to a certain limit This limit of about 4000 units of convertin per ml of plasma is produced in the experiment when the concentration of human brain extract in the final mixture is above 20 per cent Without the addition of tissue thromboplastin a convertin concentration of only about 500 to 600 units per ml of plasma is formed This finding is suggestive of a stoichiometric relationship between thromboplastin and convertin There is no linear relationship however the increase in convertin concentration being more rapid in the lower concentration range of thromboplastin and as mentioned with higher concentrations of thromboplastin the convertin concentration reaches a maximum The convertin formed is rapidly inactivated probably caused by the presence of an anticonvertin

It has been demonstrated previously that only a part of the proconvertin present in plasma is activated to convertin during spontaneous clotting of normal blood Figure 54 illustrates that

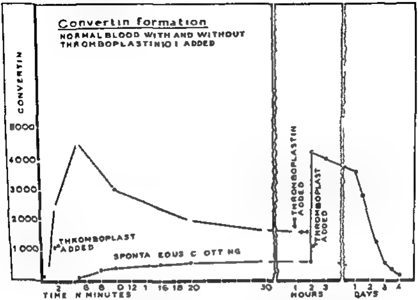

FIGURE ▪ Convertin formation with and without added thromboplastin

during spontaneous clotting of normal blood only about 500 to 600 units of convertin are found corresponding to the activation of about 10 per cent of the proconvertin present On the addition of thromboplastin to whole blood immediately after it is withdrawn (in this experiment a large excess was used) about 5000 units of convertin are formed The residual amount of proconvertin in serum after spontaneous clotting may also be transformed to convertin by the addition of thromboplastin as shown in the diagram and about the same maximum of activity is obtained

By adding small amounts of thromboplastin to serum at intervals as shown in Figure 55 we may produce convertin in steadily increasing amounts up to a certain limit When proconvertin has been completely transformed to convertin renewed addition of thromboplastin has no effect

The quantitative relationship between proconvertin and the convertin formed is illustrated by the experiment shown in Figure 56 Various proconvertin concentrations were obtained by using various dilutions of normal plasma When the thromboplastin concentration is kept constant as in this experiment the maximal convertin activity increases with increasing amounts of proconvertin in a way similar to that found when proconvertin was kept constant and thromboplastin varied

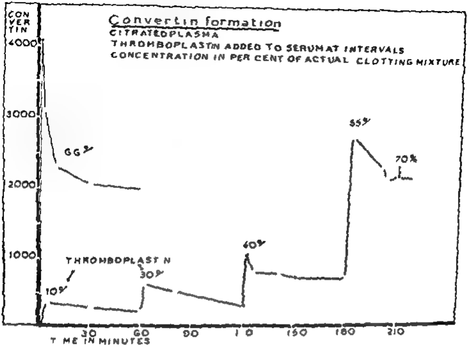

FIGURE [illegible] Convertin formation in serum by addition of increasing amounts of thromboplastin

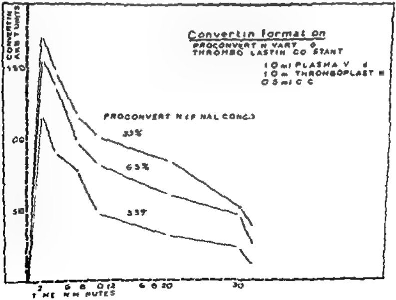

FIGURE 56 The quantitative relation between proconvertin and convertin.

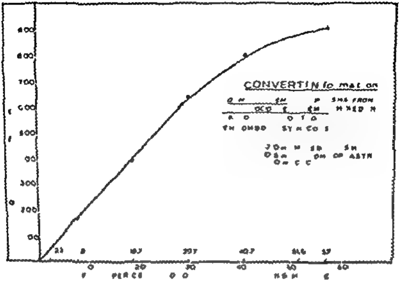

FIGURE 57 The quantitative relation between proconvertin and convertin

In the experiment of Figure 57 various proconvertin concentrations were obtained by mixing normal plasma and plasma from hypoproconvertinemia in several proportions The diagram illustrates the maximum amount of convertin formed in the presence of a constant concentration of thromboplastin The thromboplastin concentration is 14 per cent of a human brain extract in the reaction mixture In the medium concentration range an approximately linear relationship is demonstrated between the amount of proconvertin and the convertin formed

Based on these findings I may illustrate my present view on the clotting process by the scheme shown in Figure 58 (I shall not here discuss the first reaction the formation of thromboplastin by the interaction of a platelet factor and the substance which probably is lacking in hemophilia) Thromboplastin and proconvertin in the presence of calcium react together producing an active principle which I have termed convertin I stress the fact that both thromboplastin and proconvertin have a decisive influence on the maximal amount of convertin formed It is not possible at the present time to say whether one of these factors is the main substrate and the other the activator for the formation of convertin Both factors have to be present simultaneously

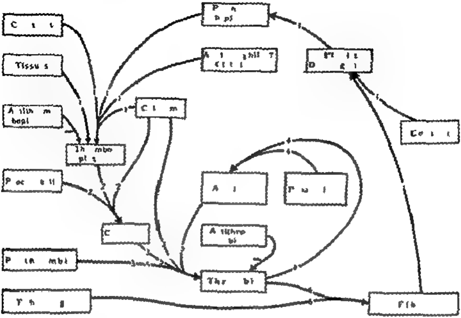

FIGURE 58 The coagulation theory

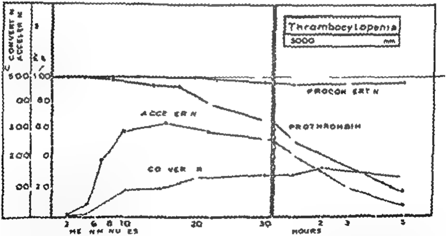

FIGURE 59 The formation of convertin and accelerin in thrombocytopenia

Convertin together with calcium brings about a slow transformation of prothrombin to thrombin Thrombin then activates proaccelerin to accelerin which speeds up the reaction

By using various quantitative methods we may follow the clotting process in pathological conditions In thrombocytopenia (Fig

ure 59) it is found that the formation of convertin takes place more slowly than in normal plasma because of the reduced formation of active thromboplastin in such plasma Accelerin formation on the other hand takes place at about the normal rate a finding which indicates that only small amounts of thrombin are necessary for the activation of proaccelerin to accelerin Prothrombin decreases at a much slower rate than during the clotting of normal blood

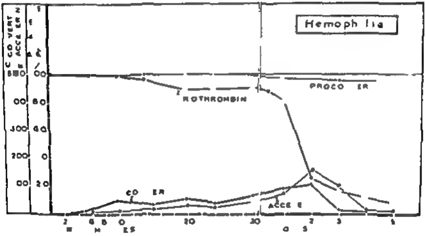

FIGURE 60 The formation of convertin and accelerin in hemophilia

In hemophilia (Figure 60) the convertin formation is limited reaching a maximum (in this patient) of only 100 units as against about 500 units in normal blood This lowered convertin production is caused by the lack of active thromboplastin Correspondingly about 90 per cent residual proconvertin is found in serum The activation of proaccelerin is also slow because of the minimal thrombin formation The slow thrombin formation is reflected in the very slow disappearance of prothrombin

In the patient with hypoproconvertinemia the disappearance of prothrombin takes place more slowly than normal It is rather surprising however that in this patient with only 3 per cent of the normal proconvertin level there is such a rapid disappearance of prothrombin as is shown in Figure 61 The rate of prothrombin consumption is only slightly reduced as compared with normal blood The convertin formed as was shown previously is very low

Alexander I am grateful for the opportunity of discussing Dr Owren s work on the proconvertin convertin system and of pre

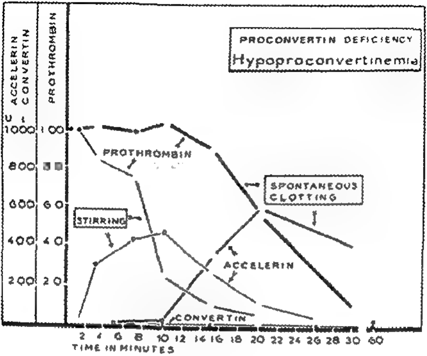

FIGURE 61 Prothrombin consumption " convertin and accelerin formation in hypoproconvertinemia

senting some of our recent work and a brief review regarding prothrombin conversion factors

In 1948 we (9) reported on a factor in normal human serum which accelerated the conversion of prothrombin by thromboplastin and calcium For want of a better name and because not enough was known about its origin its properties or its particular role in the clotting mechanism the term *Serum Prothrombin Conversion Accelerator* (spca) was used to designate this entity Subsequent work (10 11 12 13 14) regarding its separation purification its properties and the conditions under which it is elaborated from its precursor in plasma during normal or pathological coagulation indicated that the entity was a new clotting factor distinct from Ac globulin or any of the other hitherto clearly delineated factors Its possible role in the pathogenesis of defective coagulation and hemorrhage on the one hand or of excessive intravascular coagulation on the other had been alluded to (15 16)

More convincing evidence was recently provided (17) by exhaustive study of a unique patient with hemorrhagic phenomena in whom the only demonstrable coagulation defect was a deficiency or de

rangement in the spca mechanism The abnormality apparently congenital in origin could be corrected only by replacement of the missing entity It was particularly gratifying to have heard Dr Owren s observations on his case of hypoproconvertinemia for they match almost precisely our reported findings His subject constitutes the second case of this hitherto unrecognized hemorrhagic disorder

Just as Dr Owren was able to contrast his findings in hypoproconvertinemia with those of his patient with parahemophilia (Ac globulin deficiency) so were we able to compare our spca deficient patient with three siblings of another family having parahemophilia As a result of our studies reported in detail elsewhere (17 18) and as is evident from Dr Owren s observations certain unequivocal conclusions may be drawn Spca and its precursor (convertin and proconvertin) are distinct from serum and plasma Ac globulin Moreover the data provide further convincing evidence that Ac globulin labile factor factor V and the factor of Fantl and Nance are identical Deficiency of Ac globulin or of spca cause coagulation defects which simulate hypoprothrombinemia clinically and in many laboratory respects They thus constitute disorders which may properly be labeled pseudohypoprothrombinemia

The following data demonstrate some of the pertinent points

In Table V are summarized the salient clinical and laboratory features of our case of spca deficiency and our three cases of parahemophilia

Figure 62 shows the retarded prothrombin consumption in a parahemophilic and its rectification together with correction of the abnormal bleeding and clotting times by transfusion with fresh citrated blood

Figure 63 demonstrates again the retarded prothrombin consumption in parahemophilia and also retarded agglutination and disappearance of the platelets Note however the increase in one stage prothrombic activity shortly after the blood is shed exactly as is observed in normal blood This is attributable to spca formation from its precursor in plasma early in the coagulation sequence This one stage prothrombic hyperactivity , reported earlier by Langdell *et al* (19) has long been used by us to detect chemical coagulation before there is distinct evidence of prothrombin disappearance or fibrin deposition It permits evaluation of the efficacy of various procedures designed to inhibit the early phase of clotting This phenomenon cannot be due to transformation of plasma Ac globulin (proaccelerin) into the serum type (accelerin) since the patient was practically devoid of Ac globulin Moreover no such

Table V

Salient Clinical and Laboratory Features of Parahemophilia and Congenital Spca Deficiency

	FAMILIAL PARAHEMOPHILIA		CONG SPCA DEFICIENCY	
Clinical	M G	A G	J G	M R
Hemorr phenom	+++	?	0	+++
Other defects	0	+*	0	0
Type of bleeding	Ecchymoses muc memb spont or posttrauma	—	—	Same plus hemarthroses
Laboratory				
Cap fragility	Normal	—	—	Normal
Platelets	Normal	Normal	Normal	Normal
Bleeding time (min)	24 32 + (2 5)†	24	25+	12 5
Clotting time (min)	39 45 (7 14)†	36	31	14 17
Clot retraction	Normal	Normal	Normal	Normal
Proth time				
Whole plasma	54 67 (15 16)†	48	45	62 93
Dil 1 10 with normal $BaSo_4$ plasma	32-46 (26 30)†	37	43	120+
Proth units per ml				
Without Ac glob suppl	<5	<5	<5	170 120
With Ac glob suppl	100 188 (200-300)†	168	180	200 225
Ac glob activity per cent of normal	<5	<5	<5	70

(Continued on page 115)

	M G	A G	J G	M R
Labile factor activity per cent of normal	<5	<5	<5	70
Prothrombin consumption	Retarded	Retarded	Retarded	Normal
Spca formation				
During spont coag	Good	Good	Good	Poor
During storage of plas	Good	Good	—	Poor
Platelet agglut dur coag	Retarded	—	—	Normal
Effect on clotting defect				
Whole norm plasma	Completely Corrective			Partly Corrective
$BaSO_4$ norm plasma	Completely Corrective			Partly Corrective
Dicum plasma	Complet Correct	—	—	Partly Corrective
$BaSO_4$ dicum plasma	Complet Correct	—	—	Inert
Seitz filtered norm plas	Corrective	—	—	Inert
Normal serum	Inert	—	—	Completely Correct
Spca	Inert	—	—	Completely Correct
Vit K	1 0 gm K_1 Inert	—	—	Water sol Vit K derivatives ineffect
Transfusion				
Blood	Corrective 24 hrs	—	—	Of doubtful value
24 hour old serum	—	—	—	Corrective but rapidly disappearing

Epidermolysis bullosa congenitalis Also de Vries *et al* have reported on familial parahemophilia associated with syndactylism (28)

† Values in parentheses are the normal values obtained in our laboratory

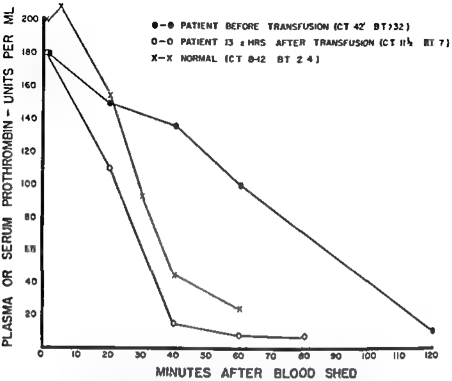

Figure 62 Prothrombin consumption before and after transfusion in parahemophiliac M G Prothrombin in plasma and serum determined by modified two-stage method in which optimal Ac globulin is provided by admixture of bovine BaCO serum (1 150 dilution) Reprinted by permission from Alexander B and Goldstein R Parahemophilia in three siblings (Owren's disease) a clinical and laboratory study elucidating certain plasma components affecting prothrombin conversion *Am J Med* (In press)

change could be induced by adding thrombin These observations are in complete agreement with Dr Owren's findings that convertin evolves normally from proconvertin in the clotting of parahemophilic blood May I add that this phenomenon is independent of the fibrinogen fibrin mechanism since it has been observed in shed afibrinogenemic blood which is incoagulable

In contrast are the observations in Figure 64 showing the sequence of events in freshly shed spca deficient blood Spca evolution and prothrombic hyperactivity do not appear These again are in agreement with Owren's experiments in hypoproconvertinemia Note however that despite the clotting defect the platelet agglutination and disappearance and the prothrombin consumption are normal thus indicating that the disturbance in prothrombin conversion as evidenced by the markedly elevated one stage prothrombin time must be in the very early phase of prothrombin activation

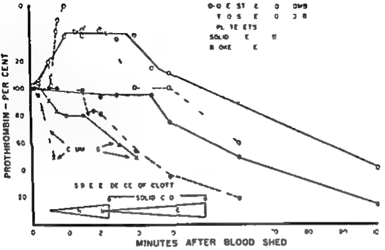

FIGURE 63 Changes in one-stage and two-stage prothrombic activity platelet number and clumping and visible fibrin deposition in freshly shed parahemophilic (M G) blood compared with normal blood On the ordinate is also plotted the platelet number (in per cent of the original which is 100 per cent) Reprinted by permission, from Alexander B and Goldstein R Parahemophilia in three siblings (Owren s disease) a clinical and laboratory study elucidating certain plasma components affecting prothrombin conversion *Am J Med* (In press)

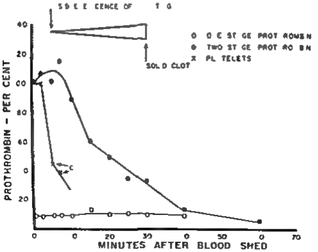

FIGURE 64 Changes during spontaneous coagulation of spca-deficient blood comparable to observations in Figure 63 In Figures 61 and 63 blood was obtained with silicone coated syringe Arquad (Armour) coated needle and then placed in glass tubes at 37 C Anticoagulant (oxalate) added at specified intervals

The elaboration of normal amounts of spca in clotting parahemophilic blood and its failure in spca deficient blood was demonstrable in several other ways, recorded in detail elsewhere (17 18 20)

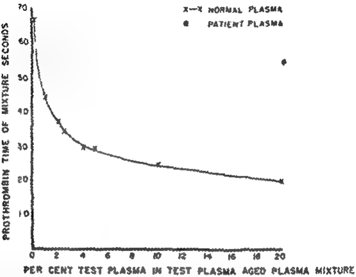

FIGURE 65 Labile factor activity of parahemophilic (M C) plasma compared with normal plasma Test plasmas were mixed with aged normal plasma in concentrations indicated

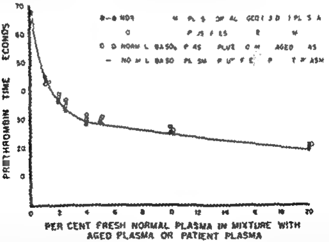

FIGURE 66 Rectifying effect of normal plasma on the retarded prothrombin conversion of fresh parahemophilic plasma (M C) or of aged normal plasma Reprinted by permission from Alexander B *et al* Congenital spca deficiency a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions *J Clin Investigation* 30 596 (1951)

Figures 65 and 66 show the reduced labile factor activity in parahemophilia It is evident that parahemophilic plasma is indistinguishable from aged normal plasma insofar as the rectifying effect on the prothrombin time of added fresh normal plasma is concerned

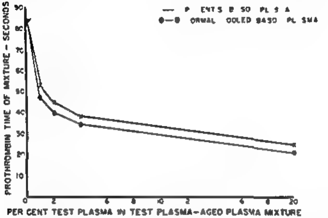

FIGURE 67 Labile factor activity of spca-deficient plasma compared with normal plasma Reprinted by permission, from Alexander B *et al* Congenital spca deficiency a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions *J Clin Investigation* **30** *596 (1951)*

Again in contrast (Figure 67) spca deficient plasma corrects the retarded prothrombin conversion of aged normal plasma indicating normal labile factor activity Moreover the spca deficient plasma corrects the clotting defect of parahemophilic plasma

Figure 68 shows two stage prothrombin determinations on parahemophilic plasma with and without supplements of Ac globulin in the form of $BaCO_3$ adsorbed bovine serum Transfusion with normal blood clearly rectifies the defect as demonstrated years ago by Owren in his original case

The observations in Figure 69 showing two stage prothrombin conversion in spca deficiency are somewhat at variance with Dr Owren s He has just shown that the plasma of his patient with hypoproconvertinemia could not induce thrombin formation from purified prothrombin despite ample amounts of A globulin Two stage prothrombin conversion in our subject was essentially normal An abnormality could be detected only when the thromboplastin used had been purified according to the technique of Dr Seegers and here the abnormality was that of retarded activation of the

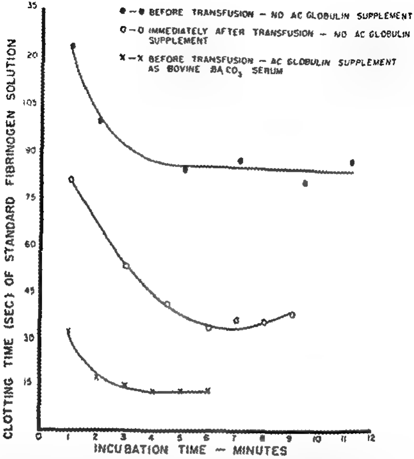

FIGURE 68 Two-stage prothrombin conversion in parahemophilic M G Ac globulin supplemented as a 1 150 dilution of bovine BaCO adsorbed serum Reprinted by permission from Alexander B and Goldstein R Parahemophilia in three siblings (Owren's disease) a clinical and laboratory study elucidating certain plasma components affecting prothrombin conversion *Am J Med* (In press)

prothrombin rather than a deficit in the final thrombin yield This *finding compatible with the relatively normal prothrombin con*sumption in the whole coagulating blood also indicates that spca functions in the early activation of prothrombin Perhaps equally important the data indicate a distinct difference in the properties of crude versus purified thromboplastin which suggests that the crude *material is* contaminated with spca Perhaps the discrepancy between our results and Owren's is referable to differences in the degree to which proconvertin (spca precursor) was reduced in our respective patients

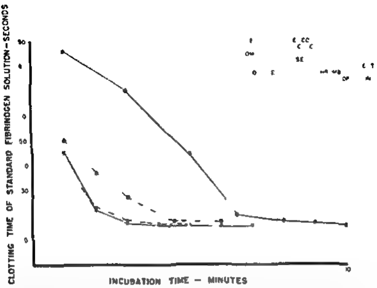

Figure 69 Two-stage prothrombin conversion in spca deficient plasma Effect of crude compared with purified thromboplastin Reprinted by permission from Alexander B Ac globulin et spca deux facteurs plasmatiques de la conversion de la prothrombine Etude et revue cliniques et biologiques Rev hemat 7 169 (195_)

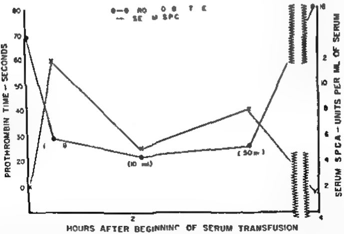

Figure 70 Effect of normal serum (24 hour old) transfusion on prothrombin time and serum spca activity in spca deficiency Figures in parentheses represent the amount of serum infused during the course of the infusion Reprinted by permission from Alexander B et al Congenital spca deficiency a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions J Clin Investigat on 30 596 (1951)

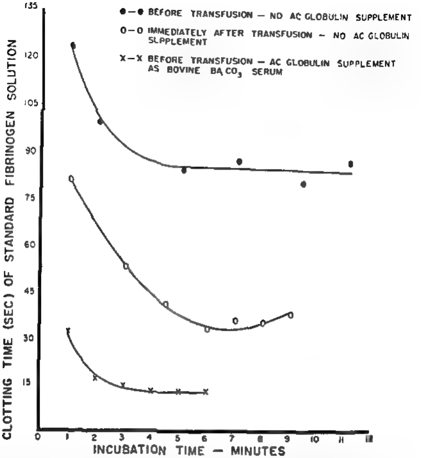

Figure 88 Two stage prothrombin conversion in parahemophilic M G Ac globulin supplemented as a 1 150 dilution of bovine BaCO adsorbed serum Reprinted by permission from Alexander B and Goldstein R Parahemophilia in three siblings (Owren s disease) a clinical and laboratory study elucidating certain plasma components affecting prothrombin conversion *Am J Med* (In press)

prothrombin rather than a deficit in the final thrombin yield This finding compatible with the relatively normal prothrombin consumption in the whole coagulating blood also indicates that spca functions in the early activation of prothrombin Perhaps equally important the data indicate a distinct difference in the properties of crude *versus purified thromboplastin* which *suggests* that the crude material is contaminated with spca Perhaps the discrepancy between our results and Owren s is referable to differences in the degree to which proconvertin (spca precursor) was reduced in our respective patients

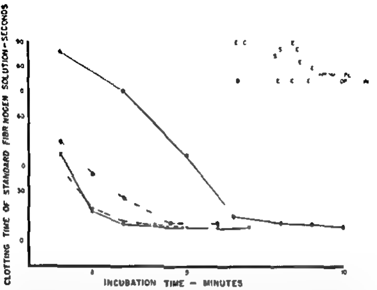

FIGURE 69 Two-stage prothrombin conversion in spca deficient plasma Effect of crude compared with purified thromboplastin Reprinted by permission from Alexander B Ac globulin et spca deux facteurs plasmatiques de la conversion de la prothrombine Etude et revue cliniques et biologiques *Rev Hémat* 7 169 (1952)

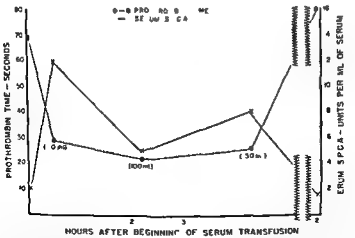

FIGURE 70 Effect of normal serum (24 hour old) transfusion on prothrombin time and serum spca activity in spca deficiency Figures in parentheses represent the amount of serum infused during the course of the infusion Reprinted by permission from Alexander B *et al* Congenital spca deficiency a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions *J Clin Investigation* 30 596 (1951)

Figure 70 shows the beneficial effect of transfusion with normal human serum in spca deficiency Note the drop in prothrombin time and elevation in serum spca activity Plasma appeared less effective This phenomenon could be duplicated *in vitro* by purified spca fractions as well as by whole normal serum but not by serum from which spca has been removed by $BaSO_4$ adsorption

Again in striking contrast, serum or spca was inert on the retarded prothrombin conversion of parahemophilic blood unless Ac globulin was simultaneously provided to the system (18) This confirms our earlier observations that for spca to act on prothrombin conversion some Ac globulin must be present (11 13) Dr Seegers experiments with our purified spca also substantiate these findings

The availability of parahemophilic plasma provided an excellent opportunity for re examining more precisely the quantitative relationship between Ac globulin concentration and the velocity of thrombin formation (as measured by the prothrombin time) in a physiologic system comprising whole plasma The data in Figure

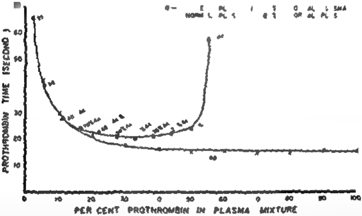

Figure 71 Curve relating prothrombin times with concentrations of prothrombin and Ac globulin Prothrombin concentrations in mixtures were calculated from two stage prothrombin determinations on the normal plasma and on the parahemophilic (M G) plasma Ac globulin concentrations were calculated on the basis that the whole normal plasma and BaSO normal plasma contained 100% Ac globulin The parahemophilic plasma was assumed to contain no Ac globulin Reprinted by permission from Alexander B and Goldstein R Parahemophilia in three siblings (Owren s disease) a clinical and laboratory study elucidating certain plasma components affecting prothrombin conversion *Am J Med* (In press)

71 demonstrate how the prothrombin time becomes increased as the Ac globulin is progressively decreased below 70 per cent of normal In these experiments however the prothrombin concentration was simultaneously altered Figure 72 shows the quantitative

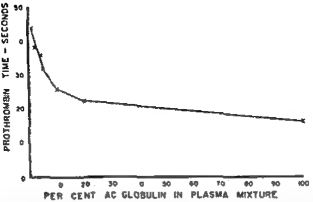

FIGURE 72 Curve relating prothrombin times with varying concentrations of Ac globulin in plasma mixtures containing a relatively fixed prothrombin concentration (50-54% of normal calculated from two-stage determinations) The parahemophilic (M G) plasma and normal plasma were assumed to contain none and 100% Ac globulin respectively Reprinted by permission from Alexander B and Goldstein R Parahemophilia in three siblings (Owren's disease) a clinical and laboratory study elucidating certain plasma components affecting prothrombin conversion *Am J Med* (In press)

relationships when the prothrombin level was maintained relatively fixed at 50 to 54 per cent of normal Note the similarity in configuration of this curve with the well known one which relates prothrombin time with prothrombin concentration

It is evident that deficiency of Ac globulin or of spca and its precursor constitute clotting defects associated with pathologic bleeding and the simulation of hypoprothrombinemia It would now seem appropriate to reclassify the general hypoprothrombinemias as follows

General Hypoprothrombinemias

- I True hypoprothrombinemia
  - A Congenital ? familial
  - B Acquired
    - 1 Idiopathic
    - 2 Liver Disease
    - 3 Vitamin K deficiency etc
- II *Pseudohypoprothrombinemia*
  - A Ac globulin deficiency
    - 1 Congenital ? hereditary
    - 2 Acquired liver disease purpura fulminans
  - B Spca deficiency
    - 1 Congenital
    - 2 Acquired dicumarol poisoning

Ac globulin deficiency may be acquired and has been observed in liver disease (21 22 23) scarlet fever (24) and leukemia (25) or it may be congenital in origin Our three patients make a total of eight recorded cases of the congenital form (26 27 28 29) Dr Owren informs me that he has seen others

Spca deficiency may also be congenital or acquired Thus far I know of only two cases of the congenital form Dr Owren s and ours In 1947 Quick (29) reported on two brothers with elevated prothrombin times which he attributed to deficiency of "prothrombin A Unfortunately these patients were not exhaustively studied by simultaneous one and two stage prothrombin determinations as well as by measurements of plasma Ac globulin by the two stage procedure and serum spca Quick labelled this condition pseudo hypoprothrombinemia but insufficient data are available to permit precise diagnosis of the clotting defect of these subjects

Spca deficiency is also associated with the use of dicumarol especially when the hypoprothrombinemia is marked (12) Mann and his colleagues have reported that co thromboplastin (which is now considered identical with spca precursor or proconvertin) is reduced by dicumarol (30) Preliminary observations in our laboratory suggest that spca precursor is reduced by phenylindandione There can no longer be any doubt however that these drugs in prothrombinopenic doses do not affect Ac globulin since plasma from patients receiving these drugs is fully capable of rectifying the defect of parahemophilic blood (18)

In the light of these advances in our knowledge cases designated earlier as idiopathic or congenital hypoprothrombinemia must be re evaluated

Some of the known biochemical and physiological properties of spca and its percursor may be briefly summarized as follows

1) Spca is relatively stable Both in serum and in purified form it is essentially unaltered (in liquid form) for at least 4 hours at 37 C (11 13) Approximately 70 per cent of the original activity in serum is demonstrable after 11 days of storage at refrigerator temperature This is not entirely in agreement with Dr Owren s data which indicate a somewhat greater lability of convertin in serum

Dried human plasma and dried serum * both more than eight years old and devoid of Ac globulin are good sources of spca precursor and spca respectively Lyophilized preparations of purified

* Stockpiled during World War II for the Canadian Armed Forces and generously provided to us by Dr A Fisher of the Connaught Laboratories Toronto Canada

spca maintain full potency for at least twelve months at refrigerator temperature

2) Spca and its precursor are adsorbable by $BaSO_4$ and $BaCO_3$ and elutable by citrate Ac globulin is not adsorbed $BaSO_4$ has a greater affinity for spca than for prothrombin however (13) Spca is also removed by Seitz filters (11 13)

3) Spca and its precursor are not precipitated from serum or plasma respectively at pH 5 0 5 2 (30) or 5 7 (11) at ionic strength one tenth that of plasma Under these conditions Ac globulin is precipitated

4) Spca uniformly can be quantitatively separated in a fraction containing less than 20 mg protein derived from 100 ml of serum Our purest preparations indicate that it exists in serum in less than 15 mg protein per 100 ml Fractions have been repeatedly obtained which are practically devoid of thrombin prothrombin or Ac globulin (13) They are also devoid of proteolytic activity

5) The ultraviolet absorption spectrum of purified spca differs from that of serum Ac globulin or prothrombin (13)

6) Spca accelerates the conversion of prothrombin to thrombin in the presence of thromboplastin Ac globulin and calcium (11 13)

7) Spca accelerates the coagulation of normal blood heparinized blood hemophilic blood thrombocytopenic blood and blood exposed to siliconized surfaces (11 13)

8) Spca is elaborated from its precursor early in the coagulation sequence frequently before there is detectable prothrombin disappearance and while substantial amounts of Ac globulin disappear (16 20 31)

9) Spca elaboration is increased when coagulation is speeded by mechanical agitation and defibrination or by thromboplastin supplements (12) Conversely retarding coagulation by removal of platelets by employing siliconized surfaces or by anticoagulants reduces the amount formed (12 15 16)

10) The conversion of spca precursor to spca is not mediated by thrombin (18 20)

11) Spca is slowly evolved in whole plasma during storage or more quickly in prothrombin rich plasma fractions obtained by $BaSO_4$ adsorption and citrate elution

12) Spca is higher in canine than in human serum lower in bovine serum (11 12 13)

13) Compared with Ac globulin purified spca is relatively inert with purified prothrombin in the isolated system in the absence of Ac globulin (Figure 73) (13) When however purified thrombo

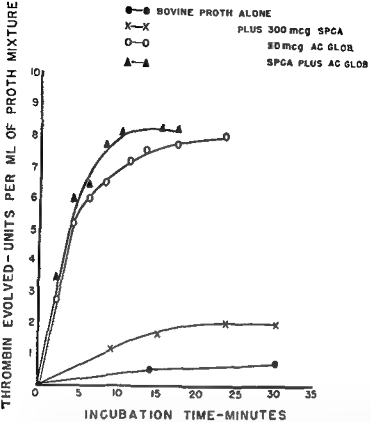

FIGURE 73 · Effect of spca and Ac globulin on conversion of purified bovine prothrombin in two-stage system Prothrombin mixture contained 50 µg of prothrombin per ml in 0 9% sodium chloride solution to which was added Ac globulin and/or spca as indicated To 1 0 ml of this mixture were added 3 0 ml of a reaction *mixture comprising 1 0 ml crude beef lung thromboplastin extract 0 67 ml of 0 025* M CaCl [redacted] 17 ml of imidazole buffer and 1 17 ml of 0 9% sodium chloride solution The evolved thrombin in the ensuing mixture kept at room temperature was measured at specified intervals by adding 0 4 ml to 0 1 ml of a 1 0% solution of fibrinogen (Fraction I Armour) and determining the clotting time The observed clotting times were converted into units of thrombin by interpolation from a standardization curve derived from clotting times obtained by adding variable amounts of bovine thrombin (Parke Davis Topical Thrombin) in the same reaction mixture to the standard fibrinogen solution One unit of thrombin was consid red *that amount necessary to produce clotting in 15 seconds Only those clotting times* between 12 and 45 seconds were considered valid for calculation

Bovine Ac globulin and prothrombin were generously provided by Dr W H Seegers Similar results were obtained with human prothrombin Reprinted by permission from Alexander [redacted] Goldstein R Landwehr G The prothrombin conversion accelerator of serum (spca) its partial purification and its properties compared with serum Ac globulin *J Clin Investigation* 29 881 *(1950)*

plastin is used spca accelerates the conversion of purified human prothrombin even when optimal Ac globulin supplements are present (Figure 74)

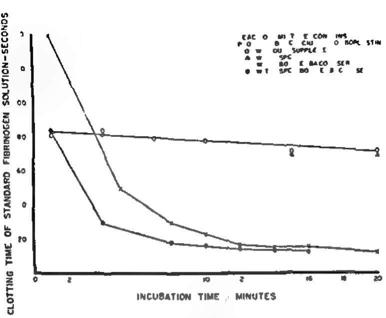

FIGURE 74 Effect of purified spca on thrombin elaboration from purified human prothrombin in the two-stage system The spca fraction was devoid of prothrombin and thrombin Bovine $BaCO_3$ serum was supplemented to the reaction mixture in the usual way as 1 150 dilution in physiological saline The thromboplastin which was used was purified bovine lung extract

Additional evidence is available regarding the role of spca in the clotting mechanism Earlier observations indicated that purified spca added to normal plasma interacts within several minutes with some component of the plasma (11 13) A similar interaction has been demonstrated in a more isolated system (32) As seen in Figure 75 a combination of washed human platelets purified human prothrombin and calcium yields thrombin extremely slowly When purified spca is incorporated into the mixture thrombin elaboration is accelerated and if sufficient time is allotted a full yield of thrombin is obtained Incubation of the platelets with spca prior to their addition to the prothrombin not only enhances the velocity of thrombin formation but also obviates the latent period of prothrombin activation This was not an artifact referable to possible slight contamination of the spca with prothrombin since in another experiment (Figure 76) a small amount of prothrombin was similarly preincubated with platelets before addition to the main prothrombin mixture Again the interaction between spca and platelets is evident whereas the phenomenon was not observed

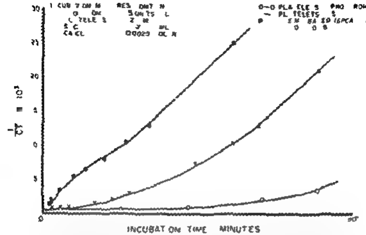

FIGURE 75 Evolution of thrombin from purified human prothrombin thrice washed discrete human platelets and purified human spca On the abscissa is plotted the incubation time (room temperature) of the reaction mixture On the ordinate is plotted the reciprocal of the clotting time (sec) times 10 of an aliquot of the reaction mixture added to a standard fibrinogen solution as in the usual two-stage prothrombin procedure 1 0 μ platelet N is roughly equivalent to 1"0 000 platelets

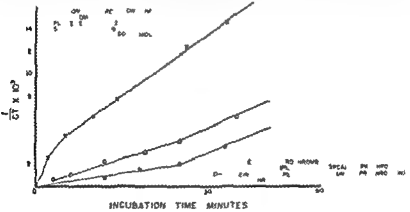

FIGURE 76 See Figure "5 Spca preparation assayed at 130 units per mg dry weight

with the prothrombin The interaction incidentally does not require added calcium The same observations were obtained when platelets were replaced with purified bovine lung thromboplastin These results are in harmony with our earlier concept that spca somehow potentiates the action of thromboplastin (12 13) a view similar to that of Dr Mann and his colleagues regarding the factor termed co thromboplastin by them

It is truly amazing and indeed gratifying to see how much in agreement with our observations are those of Dr Owren herein described There can be little question that proconvertin is identical with spca precursor convertin with spca Certain evident differences appear to be only minor and are insufficient to invalidate this concept Much work remains to be done regarding the precise role of spca and its precursor in the prothrombin conversion mechanism and their relationship to factors reported by other investigators

Seegers I should like to express my admiration for the experiments Dr Owren has presented I am a bit overwhelmed by some of them but I dare say additional pondering and further experimentation will clarify the points

The first question I have Dr Owren concerns Figure 39 In this experiment you attempted to activate purified prothrombin with proaccelerin thromboplastin and calcium but found that in the absence of proconvertin the prothrombin activated only slowly I do not understand this because when we use purified prothrombin thromboplastin and proaccelerin (plasma Ac globulin) we get rapid activation of prothrombin What is the source of your proaccelerin in this system?

Owren Proaccelerin is prepared from ox plasma filtered through pads that contain 50 per cent asbestos

Seegers If we react purified prothrombin thromboplastin from lung extract and serum Ac globulin in the form of bovine serum which has been adsorbed with barium carbonate we find that this combination activates prothrombin very rapidly Indeed I am sure the activation is as rapid as it is where you have a great deal of proconvertin in your system Furthermore if we mix purified prothrombin thromboplastin of tissue origin and platelet extract there is rapid activation of our purified prothrombin In accordance with your interpretation I would have to assume that our purified prothrombin is contaminated with proconvertin but I cannot understand why your prothrombin which is perhaps not nearly as highly purified as ours is not also contaminated with proconvertin

Warner Couldn't proconvertin be in the thromboplastin you are using?

Seegers Whether this is a possibility or not is difficult to say We centrifuge our thromboplastin wash it centrifuge it again and we can repeat this process many times and still get the same results I would imagine that it is as highly purified a thromboplastin as Dr Owren has ever used

Owren Yes There are two possibilities One is that some pro

convertin is still in your prothrombin We have repeated these experiments many times with prothrombin prepared from proconvertin free ox plasma (prepared by passing through filter pads of 20 per cent or 30 per cent asbestos) and we have always had the same results It is true that our prothrombin is a rather crude preparation

Another possibility is that lung extracts may contain convertin (activated proconvertin) Any thromboplastin containing a trace of blood may have convertin and a very small amount of this factor is sufficient to bring about a complete conversion To escape contamination we have used brain thromboplastin which has been heated to 70 C for five minutes Since the extract of human brain prepared as described in our P & P method (8) for guiding dicumarol therapy is relatively heat stable that means it does not change in activity tested on normal plasma or hemophilic plasma after being heated to 70 C for five to ten minutes

In some experiments we observed traces of other converting factors in our thromboplastin preparations especially when using lung extract and extract from placenta

Ferguson Have you used Schieffelin and Company s solu plastin which is a stable and potent prothrombin activator?

Owren No

Ferguson Prothrombin that is prepared by barium sulfate adsorption of plasma is often quite well activated by (brain) cephalin

Owren Barium sulfate adsorbs prothrombin and proconvertin

Ferguson In our work oxalated plasma is adsorbed with barium sulfate We make a 35 per cent suspension of $BaSO_4$ as follows 21 3 Gm anhydrous Na SO_4 and 36 Gm $BaCl$ 2H O are dissolved separately in minimal amounts of distilled water poured together while being stirred and centrifuged in a 250 ml bottle The precipitate is washed repeatedly with distilled water and centrifugation until the supernatant is chloride free (test with $AgNO_3$ solution) Final sediment is suspended uniformly in 65 ml distilled water One ml of 35 per cent $BaSO_4$ suspension is used for every 5 ml oxalated plasma The chosen amount of $BaSO_4$ suspension is first centrifuged to remove water then stirred with the plasma for 10 minutes at 37 C and centrifuged The adsorbate is washed first in distilled water and then in 0 9 per cent NaCl solution and finally eluted with (approximately) 0 2 M sodium citrate in one tenth the original plasma volume The citrated prothrombin solution is stored frozen at −20 C I should think this product excellent as a crude prothrombin from which a more refined reagent might be

prepared but we have not yet done this. I presume that it must be contaminated with proconvertin.

Owren: Yes. In fact, proconvertin is more easily adsorbed than prothrombin by barium sulfate. The same holds for calcium phosphate, aluminum hydroxide, and magnesium hydroxide.

Ferguson: May I ask about proaccelerin, having the same question in mind as Dr. Seegers raised a few minutes ago. Dr. Owren, is your ox plasma adsorbed with barium sulfate as well as asbestos? What makes you sure that it is completely prothrombin free?

Owren: No, we usually don't use barium sulfate adsorption of the ox plasma because by passing oxalated ox plasma once through asbestos filter pads containing 20 per cent asbestos and then once through filters containing 50 per cent, we have found that both prothrombin and proconvertin are removed completely. The possibility of prothrombin remaining in the filtered plasma can be tested by adding thromboplastin, calcium, and stored serum (which contains proconvertin) to it. If clotting does not occur, the plasma is free of prothrombin.

Ferguson: We have on occasion carefully filtered oxalated bovine plasma through Seitz asbestos pads five successive times and, on testing with calcium and thromboplastin, no clotting occurred, so that we felt sure that it was prothrombin free. Subsequently, proceeding with ammonium sulfate fractionation in order to prepare Owren's factor V, we were surprised to find that our preparation contained some active thrombin. We therefore raised the question whether we had succeeded in removing all prothrombin or whether in fact something was lacking in the test system. The last is hard to believe since accelerin should survive, as I am sure Dr. Owren will agree, and all other known clotting factors were present. If the defect is in proconvertin, it should follow, first, that this new factor is essential, and second, that it is adsorbed completely by the repeated Seitz filtration. Dr. Koller (33) recently concluded that Owren's technique can remove proconvertin while still leaving some prothrombin. Hence my question as to how sure you are that there is no prothrombin left when you use the asbestos method to get rid of it.

Owren: It depends upon the type of asbestos filter used. The point that Koller discussed in his paper is that when ox plasma is filtered through a filter containing 30 per cent asbestos, one removes proconvertin but a certain part of the prothrombin is left.

de Nicola: He used a first filtration by 20 per cent and a second filtration by 30 per cent.

Owren Yes Koller used this method of asbestos filtration to remove one of the clotting factors (proconvertin or factor VII), leaving the other factor (prothrombin) in the filtrate as we demonstrated in 1949 (4)

Wright Which Dr Koller is this?

Ferguson Fritz Koller of Zurich Switzerland

Owren Yes Koller visited me in January 1951 and became familiar with our methods At that time we used an ox plasma filtered once through 20 per cent and once through 30 per cent asbestos as a reagent for the estimation of the stable clotting factor remaining in serum As already mentioned we wrongly assumed that this factor was prothrombin but shortly afterwards March 1951 (6) we discovered its identity with our proconvertin To remove prothrombin also you have to go up to 40 or preferably 50 per cent asbestos content in your filters

Seegers I want to leave the question at this point because I don t think it can be settled by discussion

Owren No

Seegers It will have to be worked on It creates a very major difficulty in our point of view and I am sure that as we work along something will show up that will explain or at least give us a clue as to why there is this discrepancy

I should like to add that Dr Alexander sent us a sample of a plasma fraction which he had labeled spca

Alexander A serum sample

Seegers I am glad to know that I did not know it from our correspondence

We tried it out in our setup with purified prothrombin as the substrate with what we call an excess of tissue thromboplastin of lung origin optimum calcium and limited amounts of serum accelerator globulin the source of the latter being bovine serum adsorbed with barium carbonate If that is done the rate of thrombin formation in the presence of Dr Alexanders substance can be speeded up considerably If Dr Alexanders sample is comparable to proconvertin then in our system we are able to detect it by the technique just outlined I know the facts only as we found them but whether our interpretation is the correct one I cannot say

We recently came to the conclusion that during the clotting of blood or at least during the activation of prothrombin a very powerful antithrombin accelerator develops I believe that the relationship of this antithrombic activity to the activation of prothrombin is a new observation

Permit me to review briefly the fundamental concept of antithrombin which is the starting point for some of the experiments I will describe. We hold the view that thrombin may be lost due to adsorption on fibrin. In addition to the antithrombic effect of fibrin there is a cofactor in plasma and serum which together with heparin destroys thrombin and this cofactor is probably the same material recently isolated by Sylven. Besides fibrin and the co-factor there is still another antithrombin. This is undoubtedly the same as the "natural" antithrombin that Astrup has stressed as independent of heparin. We hold the view that the antithrombic substance which is independent of heparin can neutralize enormous amounts of thrombin. Indeed one milliliter of plasma can inactivate upwards of 700 to 800 thrombin units. Furthermore, this antithrombin can have its activity removed by shaking serum or plasma with ether. I mention "serum or plasma" because under certain conditions antithrombin is present in both, although in lower concentration in serum. Other investigators like Dr. Owen at the Mayo Clinic have said that the antithrombin content of serum is lower than that of plasma, but Dr. Owen in his experiments has not differentiated between the various types of antithrombin activities: fibrin, cofactor, natural antithrombin, and the antithrombin accelerator I am about to describe. In our investigations we have found that thrombin manufactured in the laboratory from purified prothrombin is stable when placed in the ether treated plasma or serum. This stability is due to the fact that the natural antithrombin is removed by the ether treatment of the plasma or serum. How do we demonstrate natural antithrombin and antithrombin accelerator avoiding the other types of antithrombin? First of all, plasma is defibrinated with a small amount of thrombin. This avoids the subsequent adsorption by fibrin of the thrombin that is added later. (The amount of thrombin used for defibrination is quite small and since it is readily inactivated in a short time by the antithrombin present in the plasma, it can be disregarded in our calculations.) For the moment we can also dismiss the heparin cofactor activity because no heparin is added. Then (Figure 77) the natural antithrombin can be demonstrated by adding calcium, thromboplastin of lung extract origin, and platelet extract to the defibrinated plasma causing the conversion of the plasma prothrombin to thrombin.

Flynn: Why is the platelet extract added?

Seegers: The platelet extract is added to furnish the equivalent of accelerator globulin.

Despite the conversion of prothrombin to thrombin, the mixture

Owren Yes Koller used this method of asbestos filtration to remove one of the clotting factors (proconvertin or factor VII) leaving the other factor (prothrombin) in the filtrate as we demonstrated in 1949 (4)

Wright Which Dr Koller is this?

Ferguson Fritz Koller of Zurich Switzerland

Owren Yes Koller visited me in January 1951 and became familiar with our methods At that time we used an ox plasma filtered once through 20 per cent and once through 30 per cent asbestos as a reagent for the estimation of the stable clotting factor remaining in serum As already mentioned we wrongly assumed that this factor was prothrombin but shortly afterwards March 1951 (6) we discovered its identity with our proconvertin To remove prothrombin also you have to go up to 40 or preferably 50 per cent asbestos content in your filters

Seegers I want to leave the question at this point because I don t think it can be settled by discussion

Owren No

Seegers It will have to be worked on It creates a very major difficulty in our point of view and I am sure that as we work along something will show up that will explain or at least give us a clue as to why there is this discrepancy

I should like to add that Dr Alexander sent us a sample of a plasma fraction which he had labeled spca

Alexander A serum sample

Seegers I am glad to know that I did not know it from our correspondence

We tried it out in our setup with purified prothrombin as the substrate with what we call an excess of tissue thromboplastin of lung origin optimum calcium and limited amounts of serum accelerator globulin the source of the latter being bovine serum adsorbed with barium carbonate If that is done the rate of thrombin formation in the presence of Dr Alexander s substance can be speeded up considerably If Dr Alexander s sample is comparable to proconvertin then in our system we are able to detect it by the technique just outlined I know the facts only as we found them but whether our interpretation is the correct one I cannot say

We recently came to the conclusion that during the clotting of blood or at least during the activation of prothrombin a very powerful antithrombin accelerator develops I believe that the relationship of this antithrombic activity to the activation of prothrombin is a new observation

would be fixed regardless of how long afterwards the aliquots were taken Nevertheless the thrombin activity is lost and indeed within approximately ten minutes it disappears completely We interpret this as indicating the formation of an antithrombin accelerator the latter appearing when the prothrombin is converting to thrombin and at the same time Ac globulin is changing

Thus we have a situation in which a mixture will not attack thrombin previously manufactured in the laboratory but does attack a thrombin which is newly formed Therefore it must be that during the first stage of clotting something is happening which permits the newly formed thrombin to be destroyed

In a third experiment we use oxalated dog serum (Figure 77) treat it with ether to destroy the natural antithrombin and then add purified prothrombin to it As before this purified prothrombin if we first convert it in the test tube by itself and offer it as thrombin to the ether treated serum will not be attacked On the other hand if purified prothrombin is added to the serum and prothrombin in the serum is activated to thrombin then in contrast to what happens when plasma is used the thrombin will remain stable for as long as three hours or more We believe that in ether treated plasma there appears an antithrombin accelerator formed during the first stage of clotting in the ether treated serum both antithrombin and antithrombin accelerator are removed

We also know that the new antithrombin is not in platelets We can add the observation that dicumarolized canine ether treated plasma to which prothrombin is added shows antithrombic activity similar to normal canine ether treated plasma

Ferguson In the control for the second experiment (Figure 77 curve labeled "Defibrinated Plasma Ether Treated) the plasma was defibrinated with thrombin and after that thrombin disappeared ether was then added?

Seegers Yes

Ferguson If a control test is then run by adding thrombin previously prepared in the laboratory that particular thrombin is quite stable in the ether treated thrombin defibrinated plasma is it not?

Seegers It is entirely stable

Flynn But nascent" thrombin is not stable

Seegers Yes newly formed thrombin is destroyed I might also say that we do this in paraffin lined test tubes That is essential

Tocantins Does the ether treated plasma prevent the conversion of prothrombin to thrombin or does it inactivate the thrombin that is formed during conversion?

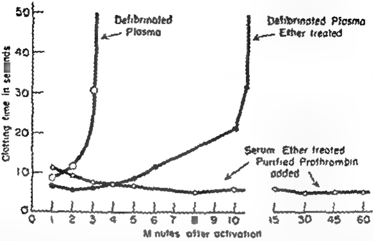

FIGURE 77 Three experiments to demonstrate antithrombin accelerator Dog plasma *is defibrinated with a small amount of thrombin The one stage technique is then* executed by adding calcium thromboplastin and Ac globulin to activate the plasma prothrombin The resulting thrombin titre is then measured many times In the case of plasma the clotting times are at first short but soon thereafter they become prolonged due to antithrombin and antithrombin accelerator The second experiment is the same except that the plasma is treated with ether before the prothrombin is activated The clotting times are short but later become prolonged despite the fact that thrombin prepared from purified prothrombin is stable in such plasma *The prolongation of the clotting time in this second experiment is due to action of anti*thrombin accelerator In the third experiment dog serum is treated with ether Purified bovine prothrombin is then added It is activated as in the other two experiments The clotting times are short and remain short because the ether treated serum is free of both antithrombin and antithrombin accelerator

of defibrinated plasma thromboplastin calcium and platelet extract has no observable end point since no fibrinogen is present An end point however is furnished by adding an aliquot of the mixture to a standardized fibrinogen and noting the time it takes for clotting to occur *We find that within three minutes the reacting mixture* contains no measurable thrombin thus indicating marked antithrombic activity A second experiment (Figure 77) consists of first defibrinating the plasma as before but then treating the plasma with ether After the ether treatment the plasma has no antithrombic activity that is to say it does not attack thrombin which is added to it However if we add thromboplastin and platelet extract to the ether treated defibrinated dog plasma it will be found that the first aliquot of the reacting mixture (removed as soon as possible) clots *fibrinogen very rapidly within five seconds* As the reaction goes on however the clotting does not remain fixed but gradually becomes prolonged *Actually we had anticipated that in this experi*ment no thrombin would be lost the clotting time of the fibrinogen

months the prothrombin activity will be less than half However when the prothrombin activity disappears another kind of activity appears an accelerator activity

I believe prothrombin itself or certainly something associated with our purified prothrombin preparations although I don't give much weight to this alternative acquires essentially the same properties which Ac globulin exhibits That can be shown in this way If a freshly prepared prothrombin sample is used as a substrate for the quantitative determination of Ac globulin one may get a certain value for the plasma concentration of Ac globulin if an old prothrombin preparation that has deteriorated in the deep freeze is used as a substrate for the quantitative determination of Ac globulin activity ten twenty and thirty times as much Ac globulin activity in the plasma may be found That prothrombin itself can undergo a change which causes it to acquire accelerator properties is the only possible explanation unless there be the other alternative which I do not believe that our prothrombin preparations are contaminated with something which acquires accelerator properties at about the same rate as that at which prothrombin activity disappears

Jensen I should like to ask whether the thrombin concentration is the same where there is an antithrombin effect and where it is not found

Seegers Yes presumably it is the same because there is a five second clotting time in both instances We have not made absolutely accurate measurements as to whether it is precisely the same but it is certain that it is close

Jensen But then wouldn't it be true that there would be a certain amount of antithrombin left even after ether treatment? It would take a certain amount of time for the completion of the reaction

Seegers When we say that there is no antithrombin left after ether treatment we mean that quite precisely

Cronkite You suggested that alteration in the Ac globulin might be associated with the antithrombin accelerator Is there much Ac globulin left after plasma is defibrinated?

Seegers Yes the small amount of thrombin we use for defibrination leaves practically all of the Ac globulin

Olwin Can you get the same thing by heating to 53 degrees and destroying the Ac globulin?

Seegers That we have not tried

Owren Do your concentrates or purified preparations of plasma Ac globulin show antithrombin activity?

Seegers The conversion of prothrombin to thrombin must be completed within the first minute I would presume because in the curve labeled "Defibrinated Plasma Ether Treated" in Figure 77 one gets a five second clot The antithrombin activity manifests itself later

Alexander Dr Seegers have I gathered correctly from your statements that serum has less of this new antithrombin activity and that this may be referable to the fact that serum Ac globulin has deteriorated under the influence of thrombin?

Seegers I believe that would be the interpretation

Alexander May I then ask as a consequence of that reasoning whether bovine serum has more or less of this new antithrombin activity than human serum this question being based on the well known fact that bovine serum retains a far greater quantity of serum Ac globulin than does human serum?

Seegers That is an interesting question One should not report on these things until one knows a little bit more about them but the situation works out in this way Plasma of bovine origin is far more active than human plasma for the antithrombin accelerator and if the experiment is tried with bovine serum which is a rich source of serum accelerator globulin the new antithrombin is not there

Allen Is there any antifibrinolytic factor in Ac globulin?

Seegers So far as we know there is no antifibrinolytic activity in accelerator globulin If anything it is the other way around The lytic principle would inactivate accelerator globulin

Warner The magnitude of this new antithrombin is sufficient to destroy purified prothrombin up to 300 units when it is added to dicumarol plasma Can it destroy more than that? Suppose normal plasma is used instead of dicumarol plasma and to it is added a large dose of bovine purified prothrombin will your antithrombin accelerator destroy the thrombin from the prothrombin originally present in the plasma as well as the added bovine prothrombin?

Seegers We have some interesting things on that but I should not like to report on them at the present time It would be too hazardous

Warner I wanted to comment about one observation made in our laboratory this time by Robert McClaughry (34) on the possible properties of prothrombin itself The best preparations of purified prothrombin of bovine origin that we have been able to get if stored in a deep freeze in physiologic saline solution gradually lose their prothrombin activity Over a period of a month sometimes two

the opportunity of reviewing this paper before publication and I continued my investigations for several months in an effort to reconcile Jacox's data with what we were learning and were interpreting not as a new factor but simply as the behavior of traces of prothrombin (and thrombin) residual in most samples of serum

Jacox added successive increments of tissue thromboplastin to serum and observed the development of a coagulant for oxalated plasma and for prothrombin fibrinogen mixtures Maximal activation was achieved within a minute and the potency then decayed rapidly Alternating decay and reactivation were observed through numerous thromboplastin additions at half hour intervals

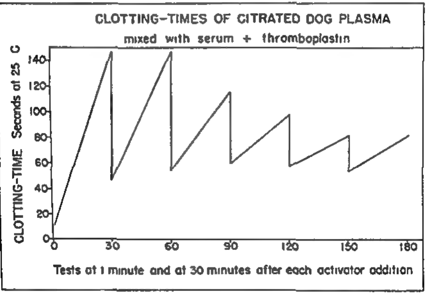

FIGURE 78

We worked with dog serum citrated plasma and brain thromboplastin Since in the multiple addition experiment of Jacox there was nothing to indicate that he took account of the increasing dilution of the serum with every thromboplastin increment we modified his technique so as to have the same amount of serum in each test using borate saline buffer to maintain constant total volumes Figure 78 illustrates the typical result Clotting times were determined at 25 C on mixing 0 5 ml of serum thromboplastin buffer and 0 5 ml citrated plasma (substrate) Tests were made

Seegers We have not tried them as yet

Lewis Does this happen only in a deep freeze?

Seegers As far as I know

Alexander Curiously enough, Dr Seegers observation with regard to accelerated prothrombin convertibility after storage is what we have found with human prothrombin fractions stored in the liquid state (14) that is to say a prothrombin fraction derived from fresh plasma will develop an ability to be converted to thrombin more rapidly as the preparation ages I think Dr Quick has also reported this observation We have always attributed it to the conversion of spca precursor present in the fraction to spca Dr Seegers interprets it as being due to an alteration in the prothrombin molecule itself

It is difficult at the moment to exclude the possibility that there is as a contaminant a trace of spca precursor or proconvertin which during storage may undergo conversion to spca the combination thus exhibiting increased Ac globulin activity if there were some Ac globulin present even in trace amounts

Seegers The difficulty with that line of thought is that it requires the assumption that the proconvertin does something to the prothrombin which causes the prothrombin to take on these properties

Alexander No I would feel that the proconvertin may be there as a contaminant converted to convertin during the process of aging and then manifesting itself as an increased velocity of prothrombin conversion in the two stage method of measurement

Seegers And you would expect the proconvertin to be activated at identical rates with the disappearance of prothrombin activity?

Alexander Certainly some alteration does take place in proconvertin during the process of aging so that there is a change That the rate of this alteration may be identical with the rate of decay of the prothrombin would be fortuitous

Ferguson I should like to illustrate with some of our own experimental data certain pitfalls in trying to devise tests to support claims for the newer clotting factors This is meant in no wise to detract from the body of evidence which Dr Owren has presented but as merely to caution all of us that before attributing certain clotting phenomena to some new factor we must make sure that all the possible implications of the old factors we have long known about have been exhausted

I happened to be doing some work with serum factors in 1949 when there appeared the first of Jacox s publications (35) I had

results It would seem therefore that we were dealing with the phenomenon of activation of traces of prothrombin persisting in dog serum The activation by thromboplastin etc is very rapid and the thrombin is subsequently removed by the progressive and rather gradual action of the normal serum antithrombin Why the repeated accessions of thrombin formation? Could it not be simply a matter of the conditions of activation? We asked ourselves what the activators were Thromboplastin was clearly adequate since we were adding a potent preparation and in cumulative amounts Its relative stability in serum was frequently demonstrated What about calcium? Jacox states that the ionized calcium in serum is equivalent to a 0 0012 M CaCl (in 0 85 per cent NaCl) solution The dog serum used in our tests analyzed 6 mg per 100 ml total calcium There were only doubtful traces (<0 2 mg per 100 ml) in the dog brain thromboplastin suspension Thus the available calcium was presumably minimal was supplied in the serum and was diluted further with each thromboplastin addition This could very well explain the clotting times in the data of Figure 79

Our next step was to determine accurately the optimal calcium level in activating the serum prothrombin with thromboplastin Having found that this was 20 millimols we now repeated the previous experiment with the same reagents but using a mixture of thromboplastin with optimal calcium instead of the thrombo plastin alone This gave a different type of result as shown in B of Figure 79 Comparing A and B in Figure 79 we note (*a*) the first 1 minute test is similar but the slight improvement (47 seconds instead of 55 seconds) does perhaps indicate a little more conver sion of the serum prothrombin (*b*) the second thromboplastin calcium addition gave very little more thrombin and subsequent additions failed to reactivate the clotting times merely becoming longer because of the progressive action of the serum antithrombin Let me reiterate that our modified technique rules out simple dilu tion effects Thus by improving the conditions with respect to available calcium we no longer get the alternate decay and re activation (35) The term reactivation is indeed a misnomer We are really dealing with additional formation of thrombin from traces of prothrombin which because of unfavorable activation conditions (i e lack of enough calcium) remain unconverted by previous additions of thromboplastin to serum

A variety of control tests accompanying our experiments further indicated that traces of calcium contributed by the serum in the presence of the strong thromboplastin additions could *weakly*

after 1 minute and after 30 minutes incubation following each thromboplastin addition The data are very similar to those in Figure 4 of the cited Jacox reference (35), namely a short clotting time soon after each addition of thromboplastin to the serum followed by considerable decay in the subsequent half hour incubation before the next addition

Jacox claimed that fibrinogen could not serve as substrate and this negative control is important for his argument With our materials however positive controls were obtained repeatedly A typical experiment using prothrombin free fibrinogen (Armours bovine fibrinogen adsorbed with $BaSO_4$) is shown in A of Figure 79 The results are essentially similar to those in Figure 78 Minor

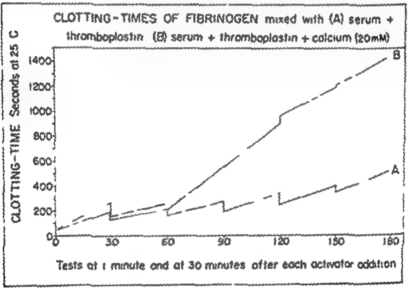

FIGURE 79

differences are that (*a*) all clotting times are longer and (*b*) subsequent thromboplastin additions are less and less able to reactivate the coagulant

That our normal dog serums contain small amounts of residual prothrombin is readily demonstrated by either the one stage or two stage prothrombin assay methods In the serum used for the tests of Figure 79 this residual prothrombin is probably of the order of somewhat less than 1 per cent of the original plasma level and could easily be ignored in insufficiently critical control tests It could however, be quite enough to explain the present series of

ing serum helps greatly if Ac globulin is deficient but not otherwise as far as we could determine Again in the presence of adequate Ac globulin Alexander's spca does not seem to add any activator effect I can only surmise in the light of Dr Owren's contributions that all our prothrombins are contaminated with practically all the convertin (or proconvertin) they need and that we shall have to develop new techniques (and Owren offers us some good leads) to prepare a convertin free prothrombin It may be recalled that we had to learn similarly how to get an Ac globulin free prothrombin and that usually this is still a relative purification (37)

Working then with the best prothrombins available to us in 1949 (most of them supplied by Dr Seegers) we did many experiments of the following type A mixture of prothrombin and various activators was incubated and sampled at intervals ranging from 15 seconds to several hours The incubated samples were tested practically simultaneously (10 seconds apart) on (*a*) prothrombin free fibrinogen and on (*b*) fibrinogen containing added prothrombin There was always surprisingly little difference perhaps slightly shorter clotting times in (*b*) with the earlier (up to 5 or 10 minutes) samples but always of the same general order of magnitude The activators included optimal calcium and thromboplastin frequently with added Ac globulin Serum (undiluted and at varying dilutions) was added to these mixtures Control tests were run on ordinary serum (containing a little prothrombin which activated typically) and on $BaSO_4$ adsorbed serum (which did not) As stated we were unsuccessful in identifying any agent in the thrombic mixture which could significantly activate the prothrombin in the test fibrinogen (*b*) At most there is some carryover of the currently recognized prothrombin activators There was no confirmation of the alleged dramatically acting factor of Jacox I believe that these remarks sufficiently summarize our experiences without need of my introducing the details of these numerous experimental tests

Wright Dr Seegers have you any comment? Dr Owren? Do you mean something has been said that everyone agrees with? This is an unusual phenomenon in this group Dr Ferguson you have broken a record

Flynn Warner and Carter have published an observation (38) in which they report that the Ac globulin activity of defibrinated plasma can be decreased by re adding the fibrinogen How does this observation correlate with the concept that plasma Ac globulin is activated to serum Ac globulin by thrombin?

Warner In the experiment to which you refer it appeared that

activate the prothrombin in citrated dog plasma

Finally we removed all traces of prothrombin from the serum by $BaSO_4$ adsorption On treatment with thromboplastin even at optimal calcium concentration it now did not yield any coagulant for fibrinogen It did so however when Seegers prothrombin was added to the serum I might just mention that minute traces of thrombin detectable in most serums even after $BaSO_4$ adsorption cannot be implicated in the phenomena I have described Certain roles of a slight amount of thrombin in some phases of the pro thrombin conversion system particularly in the conversion of proac celerin to accelerin have been noted by Dr Owren at this meeting and by Dr Seegers ourselves (36) and others at previous sessions of these Macy Foundation Conferences

I do not doubt that some of Jacox s data involve participation of serum factors which accelerate the calcium thromboplastin conver sion of prothrombin to thrombin Owing to failure I believe to give adequate consideration to the question of serum prothrombin however I find his evidence much confused and in no way com parable to the work of Seegers Alexander Owren and others Jacox s experiment (35) clearly showing that a thromboplastin serum mixture could activate the prothrombin in artificial pro thrombin fibrinogen mixtures greatly interested me But there was no control without the serum say with thromboplastin and 0 0012 M CaCl How then can anything be attributed to the serum? I was surprised that with such potent prothrombins the clotting times were not shorter Could it not be that the main phenomenon was the ordinary thromboplastic conversion of prothrombin pro ceeding at inadequate levels of calcium? Quite likely also the serum was introducing inhibitory factors further to confuse the issue We are all well aware that serum provides accelerators for the conversion of prothrombin to thrombin It is the number and nature of these accelerator factors that we are concerned with Ware and Seegers used high dilutions of serum (especially $BaCO_3$ adsorbed serum) to identify serum accelerator globulin which seems to be (or to include) the "accelerin of the new terminology contributed by Dr Owren Alexander s spca or Owren s con vertin is the other major possibility I have performed all sorts of experiments to try to demonstrate the action of spca (convertin) in the activation of purified prothrombin If there is plenty of accelerin present and we often add some of Seegers preparation to ensure this prothrombin in an isolated system is rapidly con verted into thrombin by optimal thromboplastin and calcium Add

Ac globulin to a more active form by the addition of thrombin

Seegers There is one other angle that might help Changes from the plasma type to the serum type can be detected by using thrombin concentrations which are not sufficient to clot out any visible fibrin Unquestionable changes result that can be picked up in the kinetics activation Another thing is that the minute thrombin is added to concentrates of Ac globulin made from bovine origin changes take place and very quickly the subsequent reaction occurs in which the serum type accelerator is destroyed

I cannot believe that the fibrinogen is the key to the situation The thing that gives me most difficulty about the difference between plasma accelerator and serum accelerator is the discrepancy from one species to another Why it is that the cow the cat and the rabbit have a stable serum accelerator globulin whereas all other species so far studied have none in the serum at all That I don t understand I do know that if you want to destroy the plasma accelerator globulin in the bovine species you have to use about ten times as much thrombin as in the human species It always gives me considerable cause for wonderment as to why these species differences exist Dr Owren I believe in your thesis you propose the idea of an enzyme which will inactivate the new factors You have since I think abandoned that idea but I often wonder whether or not there might not still be something in that as a possibility

Owren As was shown in Figure 45 the activity of convertin and accelerin decreases rather rapidly in serum after having reached a maximum We may assume therefore that we have substances inactivating both accelerin and convertin and I have proposed the names antiaccelerin and anticonvertin

Seegers Do you today attach considerable weight to that possibility or is it just something of a theoretical possibility to you?

Owren I showed Figure 45 on the formation and subsequent disappearance of accelerin and convertin in serum If serum is stored for 12 to 24 hours the accelerin disappears completely If it is stored for 4 to 5 days the convertin also disappears and there is only residual nonactivated proconvertin left

Seegers Ninety five per cent of the accelerin in human plasma is gone within 15 minutes and I am inclined to think the substance that does it is thrombin However I don t know how we can prove it All we know is that if we add thrombin this happens The antithrombin accelerator effect I described earlier seems to coincide almost identically on the time scale with the loss of accelerin and if you have different rates of loss of the accelerin than we have

the fibrinogen itself did decrease the accelerator activity, and if it was removed by freezing it out, there was an increase in activity If the fibrinogen could be gotten back in solution there was again the original activity We were not able to reverse the increased activity by adding Armour's fibrinogen or fibrinogen that we had made by salt precipitation from dog plasma or human plasma but only with the material which had been separated out by freezing and thawing and only if it could be gotten back into solution Part of the time the frozen out fibrinogen did not readily redissolve but if it went back in solution nicely then it seemed to restore the status

Wright Does that answer your question?

Flynn No it doesn't

Wright I didn't think it did

Flynn How do you explain this observation?

Seegers I don't follow that experiment of Dr Warner's Indeed I don't know what to make of it

Warner If thrombin activates the plasma accelerator globulin to the serum type then activation should not occur with the taking out of the fibrinogen by freezing and thawing nor should adding the fibrinogen change it back.

Alexander Didn't you and Carter advance the theory that plasma Ac globulin was converted to serum Ac globulin by virtue of the removal of the fibrinogen?

Warner That was the basis for the suggestion that it might be that way We did not make the statement that it was

Alexander I can enlarge on that from observations we have made on a patient with congenital afibrinogemia and incoagulable blood Actually whether there was less than 10 mg per cent fibrinogen in the plasma and if so how much less nobody can say because one cannot detect or measure less than 10 mg per cent fibrinogen At least it was no greater than 10 mg per cent At any rate in this subject the plasma Ac globulin activity was normal and could be enhanced by the addition of thrombin We have pretty clear cut evidence in this unique patient born with a marked deficiency of fibrinogen that the Ac globulin concentration was not abnormally high despite the congenital absence of fibrinogen and that it could be activated in a normal manner by addition of thrombin I should also like to add that if one takes a human plasma fraction rich in Ac globulin activity and devoid of fibrinogen the addition of purified thrombin provided by Dr Seegers will enhance its Ac globulin activity markedly Thus the evidence is pretty strong that fibrinogen cannot be implicated in the conversion of plasma

Owren To clarify a point when I mentioned that we had a residual 20 or 30 units of accelerin in serum after 5 hours it must be taken into account that the maximal activity of accelerin in serum on this scale is 700 units so it is reduced to below 5 per cent of the original amount after 5 hours On the other hand the disappearance of accelerin in serum depends on how the blood is handled In thrombocytopenia the concentration of accelerin in serum is slightly higher after 5 hours than in normal blood If thromboplastin is added inactivation takes place more rapidly

The possibility of convertin interfering with our determination of accelerin is ruled out because the reagent we are using contains an excess of convertin

Seegers It leads back to the same question of whether or not we can activate purified prothrombin with essentially two substances of biological origin one having the accelerator type of activity and *the other a thromboplastic activity*

There is one viewpoint that has helped me a great deal in recent times and that is to think of blood clotting factors in three compartments one a platelet compartment second a plasma compartment and the last one a fixed tissue compartment Purified prothrombin may be taken as a substrate and any two compartments provide the necessary ingredients for its rapid activation I can abandon either the platelet compartment the plasma compartment or the tissue compartment Purified prothrombin can be prepared from the plasma compartment and activated by making a combination of the various compartments plasma plus platelets or platelets plus tissues or plasma plus fixed tissues (Figure 80)

I suppose one could bring up the point of why there is a bleeding tendency in thrombocytopenia if one can activate prothrombin in so many different ways Actually there is no conflict since all our data show us that one can go to any of the compartments and if a sufficiently concentrated form of the material it contains is prepared it will activate prothrombin Under physiological circumstances it is merely a question of whether the material from several compartments becomes available in adequate amounts

Ferguson I should like to take exception to certain of Dr Seegers statements There cannot be a complete clotting system from the plasma compartment alone Something has to be added from the other (cellular) compartments Also I do not wholly agree with Dr Seegers about the platelet contribution Much more needs to be elucidated about the plasma factor which enables platelets to function as a thromboplastic agent the platelet thromboplastin

that would be interesting We find it to disappear very rapidly

Owren After spontaneous clotting (see Figure 46 page 100) we are down to 50 per cent of the maximum activity in 15 to 20 minutes but some remaining activity lasts in this experiment for 5 hours After 5 hours we still have a slight activity of about 30 or 40 units with the method used for testing

Alexander And the method is based on the one stage determination?

Owren Yes it is the specific accelerin method previously demonstrated

Alexander So they are not strictly comparable I should like to support the data provided by Dr Seegers that within one hour after spontaneous coagulation human serum has no more than 5 per cent of the original Ac globulin activity as determined by the two stage system and that within 3 or 4 hours after spontaneous coagulation, it is virtually all gone There is a difference here in the quantitative data which may well reflect the fact that in the one stage system one measures both accelerin and convertin because one measures a *velocity* of thrombin evolution and it is known that both accelerin and convertin profoundly influence that Thus one may be measuring a multiple effect here while in the isolated system one measures mostly Ac globulin activity Some time ago we published data on the consumption of the labile factor during the course of coagulation under normal and pathological conditions (39) and there we found that Ac globulin activity or labile factor activity were virtually gone in confirmation of your original article about Ac globulin activity in man Dr Seegers within a few hours after spontaneous coagulation

Seegers In the case of the dog it was so pronounced that we had to do our centrifugation in the cold and we had to use silicon technique to be able to follow it Furthermore there is I think a sharp difference on the quantitative basis In the experiment of Patton Ware and myself (40) we used silicon technique and dogs The blood was centrifuged at high speed to remove red cells and platelets The first change which we could detect in the plasma was an altered plasma Ac globulin

I am impressed by two things one the small amount of thrombin it takes to make these changes either directly or via other substances and then how tremendously effective in certain species is the disappearance of the accelerator activity and how in other species namely the cat the cow and the rabbit it may persist for long long periods of time

only saying that I know from personal experience that I can make those combinations in the reagents and I can get rapid activation of prothrombin

Alexander I am thinking purely from the point of view of the coagulation mechanism

Seegers Surely but I would follow the same pattern of argument in answering you as I did on thrombocytopenia purpura As far as Dr Ferguson s objection is concerned I can wash the platelets ten or fifteen times with saline and I can still use them to activate prothrombin

Ferguson We get different results in our test systems Travis and I (41) used Seegers purified bovine prothrombin and Ac globulin well washed dog platelets rabbit brain thromboplastin (after Quick) and Armour s bovine fibrinogen freed from all traces of prothrombin by $BaSO_4$ adsorption The platelets were concentrated in a suspension at 1/100 the original plasma volume The very minor effect of this suspension on the progress of thrombin formation in mixtures of prothrombin thromboplastin and calcium is distinctly less than that of a 1 100 000 solution of Seegers serum Ac globulin (see Table VI)

TABLE VI

Negligible Effect of Washed Platelets as "Accelerator" of the Ca thromboplastin Activation of AcG-poor Prothrombin

T	Activators ml (dilution)	Incubation period (minutes)				
		5	10'	20'	40'	60'
1	Tpln 0 25	420	300	202	141	110
2	Tpln 0 25 Plat 0 1	390	240	80	22 4	15
3	Tpln 0 25 AcG 0 1 (0 001%)	300	140	52	25 8	20 3
4	Tpln 0 25 AcG 0 1 (0 1%)	11 4	9 4	8 0	7 8	7 6

2 5 ml thrombic mixtures (T) containing borate saline buffer (pH = 7 75) 0 1 ml prothrombin (0 1 %) 0 25 ml M/20 $CaCl_2$ and activators noted Clotting time *seconds* of 0 5 fibrogen (prothrombin free) at stated incubation periods 26 C Reprinted by permission from Travis B L and Ferguson J H *Proteolytic enzymes and platelets in relation to blood coagulation* J Clin Investigation 30 112 (1951)

Combinations with PURIFIED PROTHROMBIN		RESULTS and INTERPRETATION
Platelets	1	Ac Globulin *
Plasma	2	Thromboplastin
Platelets	1	Ac Globulin *
Tissues	3	Thromboplastin
Plasma	2	Ac Globulin
Tissues	3	Thromboplastin

* The equivalent

FIGURE 80 The anatomic location of activators of prothrombin are considered to be in 1 the platelet compartment 2 the plasma compartment and 3 the fixed tissue compartment A combination of any two can furnish adequate materials for the rapid activation of purified prothrombin A combination of 1 and 2 furnishes platelet accelerator from the platelets and as the result of the interaction of platelets and a plasma globulin the equivalent of tissue thromboplastin is furnished In a combination of 1 and 3 the platelets furnish platelet AcG and the tissues thromboplastin In a combination of 2 and 3 the plasma furnishes as a minimum plasma AcG and the tissues supply thromboplastin Thus any two compartments together can supply material for the rapid activation of purified prothrombin and two main substances (thromboplastin and Ac globulin) or their equivalent are all that need to be supplied

potentiating factor we termed it at the 1949 Macy Foundation Conference (36) Our experiments fail to detect any Ac globulin in well washed platelets Thus purified prothrombin from plasma, platelets and tissue thromboplastin, provided that the last two are meticulously freed from plasma contaminants cannot in our opinion constitute a complete clotting system

Alexander The scheme is also not quite complete in view of the fact that there can be a patient who has normal platelets normal Ac globulin normal prothrombin and yet as Dr Owren has just described in his case and as was evident in the one we reported a year ago hemorrhagic phenomena and defective clotting can be severe because of a lack of proconvertin or what we call spca precursor

Seegers I cannot quarrel with the possibility that what you find in your patient might not seem to be in harmony with this I am

Fremont Smith And they perfectly well may but it has not been proved as yet

Seegers We could certainly test it with the human because we have the necessary materials We have the human prothrombin we can get the platelets and we can get the thromboplastin

Fremont Smith The dog may be different

Seegers It may very well be

Best Ferguson used dog platelets and bovine prothrombin

Loomis Back in 1943 when we took exception to Dr Quick s original publication on two types of prothrombin I think one of the most grievous errors an error that all of us recognize even today was the mixing of species in his experiments He might not have made some of the statements he did if he had worked with the same species

Fremont Smith It is worthwhile to point out that instead of a sharp difference of data on the same experiment it now becomes perfectly clear that the experiments were not the same Possibly the differences which have already been opened up are the cause of the discrepancy in the results I am emphasizing the point because I think that so very often in the absence of an opportunity to push the thing back and forth like this you get somebody grimly saying "Well I did the same thing and got opposite results period"

Tocantins Did you both use the same fibrinogen?

Seegers I used bovine fibrinogen

Ferguson I used Armour s bovine fibrinogen

Loomis Again Dr Ferguson that was not the same fibrinogen that Dr Seegers used

Ferguson No it was not

Wright Is everybody convinced that we can only apply our experiments to the species in which they are done?

Ferguson One should be hesitant about blaming discrepancies on possible species differences Such may exist but must not be assumed Rather the possibility must be fully explored and unequivocal results obtained

Wright I don t see how you can decide what other differences you had at this point but you do know that you had a species difference

Ferguson I still don t think it is logical to assume that to be the cause of the difference

Wright I don t think anybody is assuming that

Fremont Smith I only say there is a difference There are probably technical differences in the timing aging and so forth but

Seegers If you want to come to Detroit Dr Ferguson and help me make platelets purified prothrombin and thromboplastin you can see for yourself that it will work

Fremont Smith What about the sources of platelets were they from the same animal?

Seegers Same animal – homogeneous all the way through – bovine origin We collect platelets by the pound

Fremont Smith Obviously you did not do the same experiment or you would have obtained the same results so the important thing is to find out what was the difference

Seegers We have done these experiments as I have outlined them for two years I am just as confident of those as I am that I am alive

Fremont Smith The point is that one of you is not specifying the experiment accurately either you are not describing the experiment sufficiently clearly for Dr Ferguson to understand exactly what you did or else he is doing something that he does not know about which differs from your technique

Ferguson We used dog platelets

Seegers We used cow platelets

Fremont Smith You have a difference right there haven t you?

Seegers And you used purified prothrombin as a substrate Where did you get it?

Ferguson From you

Seegers The combinations I described will work in dilute solutions as well as in concentrated solutions

Fremont Smith But apparently the dog platelets used by Ferguson act differently

Seegers I have never tried dog platelets Ours were done exclusively with bovine material We had no mixing of species

Tocantins The concentration of the platelets in the two systems might make a difference too

Seegers Oh yes indeed We use a high concentration of platelets a high concentration of thromboplastin and a high concentration of prothrombin I don t blame you for being skeptical, but to my way of thinking it is a new outlook to the blood clotting picture

Fremont Smith You are assuming that it applies beyond the cow, aren t you?

Seegers Yes

Fremont Smith Is that legitimate?

Seegers I suppose so, we think in terms of these things applying to everything else

lets and fibrin In any given thrombus the ratio of fibrin to platelets regardless of whether the thrombus occurred on the venous or arterial side depends upon several factors If a vessel is suddenly compressed or ligated only a fibrin clot results Obviously under such circumstances the stagnant column of blood contains such a small number of platelets that even if they did agglutinate before the fibrin formed they would constitute an insignificant component of the clot For the thrombus to contain a large number of platelets it is necessary for it to be formed in a moving column of blood As the blood flows past the point where the thrombus is forming platelets continue to leave the blood and adhere to the wall of the vessel Eventually large masses of platelets may result Even here however as the platelet masses form some fibrin is laid down Furthermore a clot formed predominantly of platelets and a small amount of fibrin may suddenly propagate by the deposition of a large amount of fibrin giving the original thrombus a tail which may become adherent to the wall of the vessel or may remain free to wave about in the blood stream Indeed in a location like the femoral vein the tail of a thrombus may be thirty centimeters or more in length Obviously the appearance of such a thrombus will depend upon whether the histological sections were taken through the head or the tail of the thrombus In the former platelets will predominate in the latter fibrin will predominate It must also be appreciated that the ratio of the size of the head" to the tail of the thrombus varies considerably A thrombus may have a head which is only a fraction of a centimeter in length but a tail which as I said is 30 centimeters in length In such a case the tail may break away producing a fatal pulmonary embolism When an autopsy is done clinicians sometimes seem shocked that the pathologist is unable to demonstrate the exact site of the original thrombus when actually both time and legal restrictions preclude the extensive dissection needed to uncover the exact location

To return to Dr Allen s original remarks about the aortic oysters" they were as he said thrombi composed largely of platelets Actually such thrombi are fairly commonly observed particularly over aortic atherosclerotic plaques and since they are formed in a moving column of blood it is not surprising that they consist predominantly of platelets Occasionally they do reach a sufficient size to occlude the aorta but fortunately this is relatively rare It is true that in generalized platelet thrombosis with thrombocytopenia there is a much greater tendency for platelets to agglutinate indeed the thrombocytopenia may be a manifestation of the exten

this immediately gives you something to work at

Ferguson Possibly we didn t do exactly the same thing

Fremont Smith Actually you didn t do the same thing

Seegers The importance of this to me is that above all it says very emphatically that there is not a blood clotting mechanism but mechanisms Anyone who asks What is your theory of blood clotting? can be certain that he will hear from me that I do not have one There are blood clotting mechanisms That is all I get out of it and that is enough for me

Allen I should like to bring up another point that has perplexed me and it is this Are we talking of the same mechanism when we speak of arterial clots and venous clots? The basis for this question is the occasional thrombocytopenia (60 to 70 thousand) that may occur in extensive thrombotic disease particularly when the major arteries collect large platelet thrombi upon arteriosclerotic plaques At autopsy in a patient of ours whose death resulted from pulmonary embolism and in whom there had been evidence of peripheral thrombi the difference in the types of clots on the arterial side of the circulation from those on the venous side was striking It was the difference between red and white clots It was somewhat surprising to me that in the aorta below the diaphragm huge white thrombi containing little fibrin and very few cells clung to arteriosclerotic plaques There must have been fifteen of them some of them of the size and color of small oysters and they occurred in spite of anticoagulant therapy They raised a question in my mind whether there is not a real difference in what our anticoagulant program can accomplish on the venous side as opposed to the arterial side Again in the treatment of coronary disease as opposed to thrombophlebitis is there reason to suspect that a difference in our anticoagulant program may be required depending upon the arterial or venous location?

Blaustein Dr Wright and I both saw a woman who had phlebitis and at the same time a thrombocytopenia Do you recall that?

Wright Yes There is a condition which I am sure the hematologists and pathologists here will recognize It has been called by various terms one of which is thrombocytopenic thrombosing disease Another is generalized platelet thrombosis with thrombocytopenia

Flynn Phylogenetically we differentiate between the pure agglutination thrombus composed of platelets and the clot composed largely of fibrin In the human except under rare circumstances like congenital afibrinogenemia most thrombi are a mixture of plate

there were other fragmentary data which suggested a relationship So we proceeded down to Dr Milstone's laboratory with some of our spca and said Now look here let's see you make your stuff work Perhaps we can also test our spca in your laboratory with your techniques side by side The results were most interesting We had made an error in our assay of his material we had tested his thrombokinase in a concentration far more dilute than he had used There can be no question that he has a distinct clotting factor but at present as I indicated it cannot yet be integrated into the scheme previously discussed It is a coagulation factor and due cognizance has to been taken of it It is an entity which can convert prothrombin to thrombin in the virtual absence of ionized calcium it is prepared from bovine plasma globulin and it is as far as we can determine devoid of Ac globulin It does not appear to be identical with spca or its precursor but there are some similarities which warrant further exploration I just mention this as evidence to indicate that we have not exhausted the plasma clotting factors and that there may be technical errors which may not be positively excluded until one goes to the laboratory of the man who originally worked out the thing and sees the whole performance done under his very eyes

Fremont Smith Were his directions inadequate?

Alexander It was our stupidity that was inadequate or adequate however you interpret it

Seegers That idea of a factor in plasma which together with platelets gives something that will activate or help activate purified prothrombin was very clearly expressed by Dr Milstone and I guess he quotes the prior literature of the many who proposed it even before he did We confirmed what he had said but we indicated that the activity found is the equivalent of the thromboplastin which can be gotten from tissues That definitely is unique to our laboratory We say the combination of those two gives the equivalent of thromboplastin Dr Milstone simply says it gives something that will help activate prothrombin

Wright I should like to ask a question Perhaps some of you can expound on this observation As many of you know Garfield Duncan and his co workers (44) reported a year or so ago that the administration of a high fat meal tended to reduce the clotting time This was interesting in the light of reports that had come from Norway and elsewhere regarding the decrease in thrombosing accidents on low fat diets Nevertheless when an attempt was made to repeat this in our laboratory we did not obtain the same results We did

sive withdrawal of the platelets from the blood stream by thrombotic agglutination It can also be mentioned that the thrombi in this disease are more apt to be found in the capillaries and arterioles than in the larger vessels

Barker If fibrin formation had been inhibited Dr Allen dont you suppose there would have been less tendency for the platelets to hang together?

Best Dont you get platelet clots in afibrinogenemia?

Alexander We have made studies in congenital afibrinogenemia and have found that agglutination and disappearance of platelets take place within a normal interval of time after the blood is shed so that one can justifiably conclude that these phenomena are perfectly normal in the absence of fibrinogen or at least in concentrations not exceeding 10 mg per cent

Best Cant you put a little hook inside the vessel in that case of yours to make a slight injury and get agglutination?

Alexander We wouldnt dare run such an experiment in patients of that sort

Allen In a dog if one puts a string in the aorta and vena cava there is a great difference in what forms on the little thread

Wright It has been pretty well shown that most of the anticoagulants we have discussed do reduce the stickiness of platelets as well as having an effect on the venous type clots For either type of clot the indications would be essentially the same clinically In fact it would be important to keep those platelets from being sticky and if one wants to go a step further one may speculate on the possibility of a relationship between the electrical charges and their attraction or repulsion It is true that heparin paritol and other anticoagulants increase the negative charges and at least theoretically encourage mutual repulsion of small particles including platelets

Alexander Dr Wright before leaving the subject of nonprothrombin factors involving clotting I think we ought to make mention of Dr Milstones work (42 43) on thrombokinase It is pertinent to Dr Fremont Smiths remark about making certain of experimental conditions Some months ago Dr Milstone generously provided us with a sample of his thrombokinase We made an attempt to see how similar it was to if not identical with spca We did experiments in our laboratory and found that his preparation which he had sent us was inert in our hands We were about to conclude that this was strong even convincing evidence that thrombokinase and spca were distinctly different substances but

mind that allows you to assume there must be something wrong in the way you repeated his experiment perhaps because he didn t give accurate enough information If you try to repeat somebody else s method for doing anything by merely reading what he put in the literature you will go wrong because people never describe accurately what they do they always leave out the little things

Tocantins One of the fundamental principles in studies on blood clotting is that every time a clotting mixture is prepared all steps should be done on a fresh surface throughout

Wright That is what we believe

Fremont Smith There might be many who wouldn t believe it

Olwin May I ask a question? How many here routinely use cleaning solution on their glassware? About half of you I see

Loomis We not only use cleaning solution but also 40 per cent hydroxide prior to the solution

Jaques I think those who do not use cleaning solution have had the experience that cleaning solution introduces a variable which is completely uncontrollable

Olwin The point is it is different we are working with a different surface so to speak

Wright I am glad Dr Jaques spoke up because I was afraid there was an inference impugning the reliability of people who do not use cleaning solution One may defend the position of not using it

Fremont Smith The important thing is to specify which you are doing so the other person can repeat your results

Wright And also how you get rid of the cleaning solution introduced into the system That is another problem

Dr Mann do you have some points of discussion?

Mann Two years ago I put on record before this group the work which had been done in the Clinical Laboratories of the Mayo Clinic on the effects of dicumarol and similar anticoagulants Early in the use of dicumarol therapy anomalies were noted which were hard to explain in terms of the coagulation factors recognized at that time The first such anomaly to be observed was this In testing dicumarol plasma there appear differences between thromboplastic extracts that are not apparent in testing normal plasma In other words the sensitivity of thromboplastic extracts in detecting the abnormality produced by dicumarol varies entirely apart from the variations in the activities of these extracts with respect to normal plasma This finding was reported by Hurn Barker and Magath in 1945 (45) Conley and Morse in 1948 (46) reported specific differences between thromboplastins with respect to dicumarol

not get a definite shortening in the clotting time as performed by a refinement of the Lee White method Dr Tulloch who is with us as a research fellow from Edinburgh visited Dr Duncan s laboratory While there he noted the following When Duncan and his co workers made their studies they used the same syringe washing it with water and saline but using the same syringe throughout the study *in a given case* and when they did that there was a shortening in the clotting time whereas *we had used separate dry syringes for each test* Dr Tulloch returned to our laboratory and is now getting pretty well along in a series of studies in which he can sometimes duplicate their results under identical circumstances If he uses the same syringe he frequently but not inevitably gets this shorter clotting time after fat ingestion If he uses individual dry syringes he does not What is the factor that may be responsible *for the difference? What is retained in the syringe even though it* is washed with saline or water and saline? Dr Alexander have you the answer?

Alexander Last year there was quite a bit of discussion about the amount of thrombin that goes on to glass and persists on the glass despite repeated washings It is perfectly possible that repeated sampling with the same syringe accumulates enough thrombin to induce accelerated clotting The amount of thrombin that will accelerate the coagulation of shed blood can be infinitesimal

Wright These differences are significant being a matter of minutes in the shortening of clotting time I should like to point out *that this is the second time today where differences in results appear to be dependent on variations in the technique of clotting time* tests which are generally thought to be relatively simple tests as compared with prothrombin tests In my experience the clotting time test is one of the most complex and unreliable tests Quick pointed this out long ago

Fremont Smith May I comment that this is the second instance of a completely contradictory result from the same experiment being solved when *one man had the humility to go to the other s laboratory* and say Show me how you do it I would like to ask a rhetorical question How much good work has been blocked and perhaps permanently lost because somebody who was new in the field reported something and somebody working in the field said I repeated his results and they just aint so I really think this illustrates a most basic problem in scientific research

Alexander Money and time are well spent in making the trip

Fremont Smith Absolutely but you have to have the attitude of

peculiarity of thromboplastin first mentioned Although it could not be concluded a *priori* that these two abnormalities were related there was yet another peculiar effect of dicumarol which made us hopeful of a unified explanation Thromboplastic extracts prepared from the tissues of animals treated with dicumarol have been found to be unusually sensitive in detecting the effect of dicumarol This curious observation was first reported by Bose (65) and has been confirmed by Stefanini and Blanchaer (66) and by Munro and Lupton (67) The phenomenon has been investigated extensively by three different Italian investigators Rosti (68) Perrini (69) and Panella (70) We have had no difficulty in demonstrating it ourselves (71)

I have little to add to the explanation of all the foregoing facts which was presented two years ago but I shall now frame it in more definite terms The deficiency in the conversion of prothrombin to thrombin caused by dicumarol is due to lack of a factor which we have named cothromboplastin Cothromboplastin has the specific property of reacting with thromboplastin before prothrombin and the other conversion factors enter into the reaction This role in the coagulation process is the justification for the use of the name cothromboplastin When a thromboplastin preparation has already reacted with cothromboplastin it becomes insensitive as a reagent to deficiency of cothromboplastin thus insensitive to the effect of dicumarol Such insensitivity develops to varying degrees as a result of exposure to blood in the process of preparing thromboplastin from tissue Thromboplastin from the tissue of dicumarolized animals tends to be maximally sensitive to the effect of dicumarol because there is little cothromboplastin in the blood of these animals

Tromexan and compound 63 resemble dicumarol in that they produce an effect on cothromboplastin which exceeds the effect on prothrombin except during the phase of recovery from the drug Figure 81 shows an example of the effect of tromexan The larger magnitude of the change in cothromboplastin gives some reason to suspect that it may be a limiting factor physiologically as well as in the *in vitro* test the prothrombin time Vitamin K deficiency and severe liver damage also decrease cothromboplastin as well as prothrombin but as a rule the changes in the two factors are roughly of the same order of magnitude (72) However in the maximally acute hepatic insufficiency caused by total hepatectomy in the dog cothromboplastin decreases more rapidly than prothrombin (73) On the other hand when vitamin K deficiency is rapidly produced in the rat by total external drainage of the intestinal lymph

plasma These authors demonstrated differences considerably larger than those observed by Hurn *et al* probably because they were looking for such effects The findings were a surprise although perhaps they need not have been because in the original work of Dr Link s group it was noted that the one stage test as they first performed it did not have satisfactory sensitivity in detecting the effect of dicumarol on the rabbit (47) Even these early observations were hardly consistent with the view that dicumarol simply decreases prothrombin

Actually specific determination of prothrombin by the two stage method showed that early in the course of dicumarol therapy much more prothrombin remains in the plasma than would be expected from the magnitude of the change in the prothrombin time determined using a sensitive thromboplastin This observation published by us in 1947 (48) has been independently reported by Owen and Bollman (49) Mawson (50) Felix *et al* (51) Schultze (52) Sternberger (53) Shinowara and Smith (54) Koller *et al* (55) and Biggs (56) We believe it to be a fact Evidence that the effect of dicumarol is not limited to prothrombin was also furnished by several investigations in which prothrombin itself was not measured by the two stage method These were the work of MacMillan (57) and a series of researches from Dam s Laboratory (58 59) Our conclusion in 1947 was that dicumarol did indeed decrease prothrombin but that it first diminished the conversion of prothrombin to thrombin

We felt it necessary to proceed cautiously in attributing this deficiency in the conversion of prothrombin to thrombin to a given specific factor because of the large number of factors which were being described Parenthetically I wish to state the view that there is actual evidence at the present time for the existence of at least one factor distinct from any of those considered at this meeting (60 61) Without specific evidence Felix *et al* (51) and Schultze (52) attributed the deficiency in the conversion of prothrombin to thrombin caused by dicumarol to decreased Ac globulin We however had felt justified in ruling out this particular factor as the explanation of the deficiency because Quick (62) had originally demonstrated the labile factor (synonymous with Ac globulin and factor V) in dicumarol plasma Quick and Stefanini (63) Bjerkelund (64) and Mawson (50) have presented evidence that this factor is not appreciably affected by dicumarol

We were interested in an explanation which might relate the deficiency in the conversion of prothrombin to thrombin to the

peculiarity of thromboplastin first mentioned Although it could not be concluded a priori that these two abnormalities were related there was yet another peculiar effect of dicumarol which made us hopeful of a unified explanation Thromboplastic extracts prepared from the tissues of animals treated with dicumarol have been found to be unusually sensitive in detecting the effect of dicumarol This curious observation was first reported by Bose (65) and has been confirmed by Stefanini and Blanchaer (66) and by Munro and Lupton (67) The phenomenon has been investigated extensively by three different Italian investigators Rosti (68) Perrini (69) and Panelli (70) We have had no difficulty in demonstrating it ourselves (71)

I have little to add to the explanation of all the foregoing facts which was presented two years ago but I shall now frame it in more definite terms The deficiency in the conversion of prothrombin to thrombin caused by dicumarol is due to lack of a factor which we have named cothromboplastin Cothromboplastin has the specific property of reacting with thromboplastin before prothrombin and the other conversion factors enter into the reaction This role in the coagulation process is the justification for the use of the name cothromboplastin When a thromboplastin preparation has already reacted with cothromboplastin it becomes insensitive as a reagent to deficiency of cothromboplastin thus insensitive to the effect of dicumarol Such insensitivity develops to varying degrees as a result of exposure to blood in the process of preparing thromboplastin from tissue Thromboplastin from the tissue of dicumarolized animals tends to be maximally sensitive to the effect of dicumarol because there is little cothromboplastin in the blood of these animals

Tromexan and compound 63 resemble dicumarol in that they produce an effect on cothromboplastin which exceeds the effect on prothrombin except during the phase of recovery from the drug Figure 81 shows an example of the effect of tromexan The larger magnitude of the change in cothromboplastin gives some reason to suspect that it may be a limiting factor physiologically as well as in the *in vitro* test the prothrombin time Vitamin K deficiency and severe liver damage also decrease cothromboplastin as well as prothrombin but as a rule the changes in the two factors are roughly of the same order of magnitude (72) However in the maximally acute hepatic insufficiency caused by total hepatectomy in the dog cothromboplastin decreases more rapidly than prothrombin (73) On the other hand when vitamin K deficiency is rapidly produced in the rat by total external drainage of the intestinal lymph

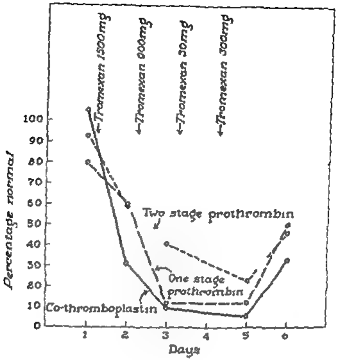

FIGURE 81

prothrombin is affected more than cothromboplastin (74) We have interpreted those results to mean that cothromboplastin has the more rapid rate of physiologic turnover of the two Thus when its formation is prevented by dicumarol or by removal of the liver it decreases more rapidly than prothrombin When however absolute scarcity of vitamin K is the limiting factor it appears conceivable that the substance with the more rapid turnover cothromboplastin might be formed more economically and for a time show the higher level

Representative samples of most of the experiments that have led us to the concept of cothromboplastin have been published (30 72 75 76) Rather than recapitulate I should like to discuss an example of a type of experiment which has not been presented previously although it has influenced us somewhat Table VII shows an example of the cothromboplastin reaction As a result of three minutes exposure of the thromboplastin to very dilute plasma (1 to 300) the subsequent conversion of prothrombin to thrombin in the usual aliquot of plasma in 1 to 25 dilution is greatly speeded Prothrombin or the labile prothrombin conversion factor are quite

TABLE VII

A Reaction Between Plasma and Thromboplastin Which Precedes the Conversion of Prothrombin to Thrombin

First reaction mixture	Time interval minutes	Second reaction mixture	Time interval minutes	Clotting time s conds
0 5 ml plasma 1 25 0 5 ml buffer 1 0 ml thrombo plastin—Ca	1	————	——	49
0 5 ml plasma 1 300 1 0 ml thrombo plastin—Ca	3	0 15 ml first reaction mixture 0 05 ml plasma 1 25	1	24
0 5 ml plasma 1 300 1 0 ml thrombo plastin—Ca	3	————	——	390
0 1 ml plasma (undil) 0 1 ml 0 025 M CaCl 0 2 ml thrombo-plastin—Ca	3	0 5 ml first reaction mixture dil 1 75 0 5 ml plasma 1 25 1 0 ml throm boplastin—Ca	1	40

* 0 2 ml of indicated reaction mixture + 0 2 ml of fibrogen acasia

unnecessary for this reaction which can be readily demonstrated with fresh aged or heat inactivated plasma or serum Even with fresh plasma only a small fraction of the small amount of prothrom bin present in the very dilute plasma is converted to thrombin If this reaction were due to a factor which forms in the plasma because of treatment with thromboplastin it would be expected that if plasma in relatively high concentration were treated with thrombo plastin one could dilute this reaction mixture greatly adding new thromboplastin and still get the effect This has not been true in our experience (last line of Table VII) The original thromboplastin which reacted with the dilute plasma or serum must remain in the system in order to demonstrate the cothromboplastin reaction

Accordingly we think of cothromboplastin as a factor which exists in plasma and remains in serum It plays its role before the conversion of prothrombin to thrombin rather than being formed

in the process Its activity is inseparable from that of the thromboplastin with which it has reacted but it is definitely separable from that of prothrombin and the labile prothrombin conversion factor In our opinion the most convincing evidence for the existence of this factor in plasma is the deficiency in the conversion of prothrombin to thrombin caused by dicumarol for this coagulation defect is unexplained except on the basis of deficiency in cothromboplastin In any case however we do wish to present all our evidence for evaluation

Two years ago Dr Jaques presented at this meeting a quotation which he wished to be off the record although some thought that it would have been salutary to have included it * I should like to submit the following quotation (77) for the record as appropriate to what happens in blood clotting

In 1658 Christiaan Huygens published in a scientific journal the following notice aaaaaaa ccccc d eeeee gh iiiiii llll mm nnnnnnnnn

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* *Editor's Note* The quotation given by Dr Jaques followed a discussion of the prothrombin consumption test and was as follows

Don Quixote and Sancho have reached the river Ebro and seeing a boat tied to the bank the Don is convinced that if he steps into the boat and casts off enchanters will immediately waft them to the Antipodes Tying Rocinante and the ass Don Quixote and Sancho step into the boat and push off whereupon Don Quixote assumes they have already crossed the equator

I may inform you Sancho he continued that one of the ways of the Spaniards who embark at Cadiz for the East Indies have of telling when they have passed that equinoctial line is this if the lice die upon every man aboard so that not a one is to be found anywhere even though they offer its weight in gold for it they then know they are past the line and son Sancho just run your hand over your thigh and if you come on anything that's alive it will remove all doubt but if you find nothing then we have made the crossing

" I don't believe a word of it declared Sancho but nevertheless I will do as your Grace bids me although I must say I don't know why we have to make all these experiments for I can see with my own eyes that we're not five yards away from the bank nor have we dropped two yards below the spot where the animals stand for Rocinante and the ass are still there where we left them and if you take a fixed point as I am doing now then I swear we're not moving any faster than an ant's pace

Make the test that I asked you to Sancho said Don Quixote and do not be bothering your head with anything else for you know nothing of colors lines parallels zodiacs ecliptics poles solstices equinoxes planets signs and points of compass these being the units of which the celestial and terrestrial spheres are composed If you know all these things or even a part of them you would clearly perceive what parallels we have crossed what zodiacal signs we have seen and what constellations we have left and are now leaving behind us But I tell you again feel and hunt for I am certain you are cleaner than a smooth white sheet of paper

"Sancho did so and having run his hand gently all the way down to the inside of his left knee he looked up at his master and said Either the test is a false one or we're not within many leagues of where your Grace says we are

How is that?" said Don Quixote Did you find something?"

"Yes a number of somethings said Sancho

Cervantes *Don Quixote* The Samuel Putnam translation New York Viking 1949 (p 700)

oooo pp q rr s ttttt uuuuu The stolid Dutch astronomer did not deign to amplify his cryptic statement until three years later Then Giovanni Domenico Cassini startled the world by announcing that he had observed tremendous disc shaped rings around Saturn Huygens was anything but impressed He commented with perhaps a slightly patronizing note in his voice that this was no new discovery He suggested that scientists re examine his scrambled string of letters that had evoked so much ridicule When properly arranged he explained they announced in Latin precisely the same finding (His statement read Annulo cingitur tenuui plano nusquam cohaerente ad eclipticam inclinato)

Wright Questions? Comments?

Tocantins Is this effect referred to analogous to the one reported for Russell viper venom? If you remember in the early days of dicumarol therapy it was found that Russell viper venom could not be used as a thromboplastin in the one stage prothrombin assay because it gave misleading results with dicumarol plasma Do you explain these results on the basis of a lack of cothromboplastin in dicumarol plasma which did not influence the action of the Russell viper venom?

Mann My suggested explanation would be that Russell viper venom does not need to undergo the cothromboplastin reaction and can convert the prothrombin of dicumarol plasma to thrombin without need of cothromboplastin Therefore Russell viper venom is an insensitive indicator of the effect of dicumarol Tissue thromboplastin however may or may not be sensitive to the effect of dicumarol depending upon the degree to which it has been exposed to cothromboplastin in the process of preparation

Flynn By sensitive thromboplastin you mean one which when used on dicumarolized plasma gives a considerable prolongation of the one stage prothrombin a prolongation over and beyond what might be anticipated from the results of the Iowa two stage prothrombin determination?

Mann Exactly Thromboplastins vary greatly in sensitivity I don t know that one has to use the most sensitive thromboplastin possible The sensitivity needed I suppose varies somewhat with the rules followed but I think that one should realize that sensitivity does vary and act accordingly

Seegers I was very much interested in your quotation of Bose who made the observation that dicumarol altered the thromboplastin content of the brain I was rather astonished that a drug could change the composition of people s brains and even more astonished

to have that as the conclusion of the paper Apparently however you would not place that interpretation on their work

Mann No I realize that you have found that work very difficult to accept and have so stated I do not think that dicumarol modifies the intracellular thromboplastic protein I think that because of the effect of dicumarol the thromboplastic activity of the extracts we prepare from tissue is different

Wright I dont follow you

Flynn Dr Mann your implication is that in the preparation of thromboplastin any contamination of that thromboplastin by blood plasma or serum influences the sensitivity of the thromboplastin?

Mann Yes we can easily demonstrate this effect with serum as dilute as 1 2400 using a highly sensitive thromboplastin I think that contamination of any thromboplastic extract to this degree would be exceedingly difficult to rule out

Flynn Your interpretation is then that in an animal who has received dicumarol the small amount of blood which inevitably contaminates the brain when thromboplastin is prepared will by virtue of the dicumarol administration lack cothromboplastin while in a normal animal the blood contaminating the brain will contain cothromboplastin therefore the former gives a different result in the assay of dicumarolized blood than the latter I must say that this explanation seems far more likely than an intrinsic change produced by dicumarol in the thromboplastin *per se*

Along this same line it should be stressed again that thrombo plastin prepared by the Quick method is always contaminated by a certain amount of blood After all the brain is a very vascular organ and the mere stripping away of the meninges as Quick rec ommends does not eliminate the blood from the vessels within the brain Quicks thromboplastin does to be sure contain less blood than lung extract but it is by no means as free of blood as Quick implies when he extols the virtues of his thromboplastic brew

Mann Exactly

Alexander In support of Manns interpretation can be cited data we presented at the Federation meetings last year (32) A serum fraction containing spca incubated with thromboplastin will show a more profound acceleration of the activation of purified human prothrombin in an isolated system than either one alone The data indicate that a serum fraction rich in spca interacts with thrombo plastin or for that matter also with platelets to produce a milieu capable of activating prothrombin

Flynn Although in my opinion we know very little about the

interaction of the many variables concerned in the activation of prothrombin to thrombin yet I must say that Mann's original data relating to the necessity of doing a preliminary reaction between thromboplastin and his serum factor is the only convincing evidence that a reaction actually occurs between the two This observation alone seems to me to justify the term cothromboplastin In fact from the standpoint of priority it should supersede convertin and spca

Mann We have demonstrated the cothromboplastin reaction using human platelet material as thromboplastin (78) I should say the principal difference between our approach and yours Dr Alexander is that we are primarily concerned with the explanation of a deficiency in the conversion of prothrombin to thrombin We are also influenced by the fact that the test we use to study this deficiency is based on the early phase of the lag period which precedes the formation of any appreciable amount of thrombin

Alexander I would concur entirely with that in fact you stated that there was early activation of prothrombin to thrombin

Mann That is what we think anyway

Tocantins Can you use this factor to explain the discrepancy between prothrombin titrations in newborn blood by the one stage and two stage methods? Is it possible that with the one stage method there is more available cothromboplastin and therefore the one stage method gives higher readings than the two-stage method which apparently is not sensitive to cothromboplastin?

Mann We feel much as you say that the one stage method tends to be sensitive to cothromboplastin (as well as to other conversion factors) but is not necessarily very sensitive to prothrombin itself while the two stage method tends to be just the reverse Recently Dr Owen has found that the addition of one of Dr Seegers highly purified prothrombin preparations (the one which proved free of conversion factors) did not affect the prothrombin time of dilute plasma (79) It appears that prothrombin is not necessarily a limiting factor in the prothrombin time The original Iowa group found that in the newborn (80) and in liver disease (81) the two stage prothrombin level may be substantially reduced while the one stage prothrombin time remains essentially normal We have confirmed this finding in liver disease (82) I am glad Dr Tocantins has brought up the matter of these discrepancies between the two stage and one stage methods since I think they were as Dr Flynn pointed out to me privately the first actual evidence for the existence of those factors with which we are now concerned

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DEFECTS IN HEMOSTASIS PRODUCED BY WHOLE BODY IRRADIATION*

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IT IS APPROPRIATE to reflect for a moment on the reasons why radiation injury is now attracting so much attention It is of relatively minor importance in everyday clinical medicine particularly so far as whole body irradiation is concerned However it becomes of great importance in military medicine and in civil defense with the realization that we can protect ourselves to a large extent given adequate warning and preparedness against the traumatic and the thermal injuries of atomic bombs Under these circumstances the correct treatment of radiation injury becomes of vital importance in survival from atomic attack

Radiation injury presents many features that are both dose and time dependent (1 2 3 4) The hemorrhagic tendency is one of these

In Table VIII is seen the correlation between dose of radiation per cent mortality mean survival time the range in survival bleeding tendency and general symptoms It is apparent that the bleeding tendency does not become marked until the mortality approaches 100 per cent As the dose of radiation is increased to the extent that survival time is drastically reduced (1000 r or more) the bleeding tendency fades away and constitutional symptoms of vomiting diarrhea and anorexia become prominent

* The work reported herein involves the coordinated efforts of the following individuals D P Jackson M D George Dillard M D F W Ulrich M D G V LeRoy M D G J Jacobs B Halpern and N R Shulman M D At all times the activities of this group have benefited from the friendly advice support and encouragement of Captain R H Draeger MC USN Rear Admiral C F Behrens MC USN Captain W E Kellum MC USN and K S Cole Ph D In addition the collaboration of K M Brinkhous and his group at the University of North Carolina and of L M Tocantins and his group at the Jefferson Medical College has been stimulating and profitable The author has been fortunate in being continuously connected directly with the studies on radiation injury and allied hematologic problems since their inception at the Naval Medical Research Institute following the initial atomic bomb tests at Bikini in 1946 Acknowledgements would be inadequate without the recognition of the loyal support of the following superb technical staff in the field and in the laboratory D C Eltzholtz HMC USN C R Sipe HMC USN P K [illegible]hork HMC USN J Cassidy HMC USN J [illegible] Freeman HMC USN and Mildred Walters

TABLE VIII

Correlation of Dose of X-ray, Per Cent Mortality, Survival Time, Bleeding Tendency, and General Symptomatology in Dogs Exposed to 2.0 Mev X-ray

Dose in r	No. Exposed	No. Dying	Percent Mortality	Survival time in days: Min	Mean	Max	Degree of Bleeding	Symptoms
150	18	0	0	—	—	—	±	Continue eating
200	11	3	27.3	12	16	21	+	
300	15	5	33.3	15	19.6	23	2+	"
350	8	4	50.0	10	14.0	20	3+	"
375	3	3	100	14	19.0	24	3+	"
400	[redacted]	22	84.6	6	17.6	40	4+	Initial symptoms rare
450	4	4	100	14	14.5	15	4+	" "
500	11	11	100	7	10.0	12	4+	50% vomit on day of exposure 4-10 day latent period
600	49	49	100	4	11.9	21	4+	
800	4	4	100	9	11.8	17	4+	" "
1000	7	7	100	3	7.0	9	2+	All vomit day of exposure 2-3 day latent period anorexia and diarrhea
1500	6	6	100	0.5	3.1	4	0	Vomiting and diarrhea from exposure to death
2000	6	6	100	3	3.8	4	0	" "
3000	6	6	100	3	3.0	3	0	" "

In general external bleeding is rarely seen before the seventh day With doses greater than 1000 r the survival time is less than seven days and accordingly external bleeding is not seen With these shorter survival times there is no purpuric bleeding during the life of the animal The cause of death apparently changes from a combination of infection hemorrhage and anemia to a fatal intoxication" presumably produced by irreversible lesions of the gastrointestinal tract (3) and characterized by intractable vomiting and diarrhea (4) At death the animals are markedly dehydrated

The historical aspects of the hemorrhagic phase of radiation injury will not be considered other than to state that conflicting views have been published The following references cover the important contributions since the discovery of radiation (1 2 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20) The reasons for the conflicting views are varied Two important ones are

a) Failure to recognize the dose dependence of many radiation effects

b) Conclusions based on inferences from indirect evidence

As a result of the uncertain nature and dubious value of therapy of the hemorrhagic tendency a long term program was initiated in which it was planned to reinvestigate all phases of the hemostatic mechanisms after varying doses of radiation

The first step was the establishment of a dose of radiation which would produce diffuse bleeding in all of the dogs This was accomplished by doses from 400 to 600 r The dose usually used in the following studies was 600 r of 2 mev x ray (300 r to both sides of the dog)

Tissue dose the amount of energy absorbed and its distribution in tissue significantly influences the response of mammals to radiation The technique of irradiation affects the tissue dose Therefore our techniques and actual measurements of ionization in phantoms of density equivalent to tissue are shown in Figure 82 The ionization at various depths is shown when irradiated from the right and the left and the summation of ionization in tissue when the dose is split half to each side of the animal Unless irradiation techniques are such as to produce uniform tissue dose throughout the animal comparison of results in terms of r in air is not possible By using the bilateral technique and with 2000 kvp x ray having a mean energy of about 540 kv uniform ionization throughout the body of the animal is produced

Wright Dr Cronkite may I interrupt for just a moment? Since many of the members of this group are not familiar with the details

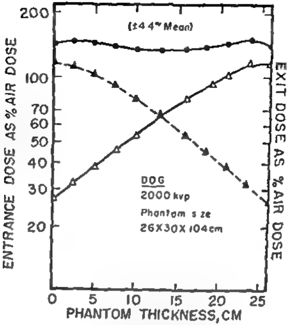

FIGURE 82 Ionization in phantoms of unit density material as per cent of air dose Solid triangles represent fall off when irradiated from left to right open triangles when irradiated from right to left Solid circles are the summation of both curves or the ionization in tissues when the dose is split half to each side of the dogs

of this work certainly not as familiar as they are with anticoagulants and clotting factors I hope they will interrupt for explanations of any points that are not clear to them Is that satisfactory?

Cronkite Certainly

Seegers What is meant by phantom density ?

Cronkite These measurements rather than being made in the animal are made in a unit density material of approximately the same electron density of tissue The actual ionization at various depths *is measured so that one gets an estimate of the absorption* of radiation in the tissue Animals of approximately the same size are used and literally cubed in a box thus making them conform roughly to the phantom dimensions

To continue with the general pathologic observations following whole body irradiation of 400 600 r it was found that diffuse bleed ing particularly into the lymph nodes alimentary canal and urinary tract occurred in 48 of 49 animals dying spontaneously The dog that did not show hemorrhage at autopsy died on the fourth day All others died between the seventh and twenty first day after irradiation External evidence of bleeding was usually seen by the

eighth day and was uniformly seen by the eleventh day In a series of 102 dogs which were killed at regular intervals from the time of exposure through the fourteenth day hemorrhage was not seen grossly or microscopically until the seventh day and appeared first in the lymph nodes Bleeding in the gastrointestinal tract was uniform by the tenth day It is emphasized that external evidence of bleeding is commonly seen by the eighth day and is uniformly seen by the twelfth day In the studies the lymph nodes were dissected out for gross and microscopic studies on the character of bleeding into lymph nodes in the irradiated dog One dog died of hemorrhage from the nose At autopsy the left frontal sinus was filled with clotted blood An ulcerative lesion in the mucous membrane was the bleeding point Hemorrhagic tonsils with and without gross ulcerations were common Retropharyngeal lymp nodes were frequently swollen with hemorrhage Bleeding into the mesenteric attachments and into mesenteric lymph nodes was most frequently observed along with diffuse petechiae of the mucosa of the whole bowel At times there was extensive hemorrhage into the bowel wall dissecting the mucosa from the muscularis When large areas were lifted up superficial ulceration frequently appeared in the central part presumably due to ischemia Bacterial necrosis of the bowel secondary to agranulocytosis was uncommon except in the rectum Bleeding into the intercostal muscles and into the diaphragm were found as were large subcutaneous hematomae

The next step was the reinvestigation of the problem of anticoagulants Holden *et al* (15) Rosenthal and Benedek (16) and our own studies were not in complete agreement with the Allen *et al* (13 14) and Jacobson *et al* (17) concept of heparinemia" as the major cause of radiation and nitrogen mustard induced hemorrhage In the work at Bikini indirect confirmation of the original observations of Allen and Jacobson on heparinemia was made In following this work further in the laboratory evidence for the presence of anticoagulants was not obtained As a result of these conflicting results the problem was reinvestigated

In the first place detection of anticoagulants is not simple It appears that the only absolute proof for an anticoagulant is its extraction and then demonstration of the anticoagulant effect in a relatively pure clotting system Our studies (21 22) consisted of a systematic daily study of platelets whole blood clotting time clot retraction Conley s nonspecific test for anticoagulants (23) and LeRoy s (24) and Allen s (25) indirect protamine assay for heparin like substances

TABLE IX A

Period Day Number	DOG No 84 Coagulability Test	L R yeast Titre PHR ratio	All Test mg Protamine	Clotting Time minutes	Platelets 10³ mm	DOG No 85 Coagulability Test	L R yeast Titre PHR	All Test mg Protamine	Clotting Time minutes	Platelets 10³ mm	DOG No 86 Coagulability Test	L R yeast Titre PHR	All Test mg Protamine	Clotting Time minutes	Platelets 10³ mm
												1 75/1			
	Neg		0 12	17 5	330			0 10	11 5	450	Neg	1 75/1	0 10	6 0	250
Control					268				11 5	300		1 75/1		13 5	370
					230				15 0	797				8 0	780
1				14 0	240				18 0	330				20 0	
2	Clot			22 0	320	Neg			750	250				20 0	235
3				22 0	210				22 0	250		1 75/1		18 0	735
4	Neg			17 0	250	Clot			14 0	267	Clot	1 75/1		18 5	345
5				18 0	2 0	Neg			18 0	275	Neg	1 75/1		25 0	.55
6	Neg			30 0	194				32 0	240		1 75/1	0 12	29 0	245
7	Clot		0 10	37 0	160	Neg		0 12	29 0	130	Neg	1 75/1	0 10	34 5	165
8	Neg		0 12	35 0	059	Neg		0 12	37 0	090	Neg	1 75/1	0 12	48 0	085
9	Neg		0 10	8 0	030	Neg		0 10	84 0	012	Clot	1 75/1		76 0	015
10	Neg		0 10	> 2 hrs	0 326	Quest		0 12	> 2 hrs	0 000	Neg	1 75/1	0 14	> 6 hrs	003
11	Neg		0 12	60 0	0 002						Neg	1 75/1	0 14	> 9 hrs	003
12	Pos		0 14	> 6 hrs	0 000										
	Death—12th day					Death—10th day					Death—11th day				

Period Day Number	DOG No 89 Coagulability Test	L R yeast Titre PHR ratio	All Test mg Protamine	Clotting Time minutes	Platelets 10³ mm	DOG No 90 Coagulability Test	L R yeast Titre PHR	All Test mg Protamine	Clotting Time minutes	Platelets 10³ mm
	Neg	1 75/1				Neg	1 75/1			
		1 75/1	0 10	13 0	360		1 75/1	0 10	5 0	180
Control				12 0	350				16 0	290
				18 0	345				14 0	285
1				19 0					20 0	
[illegible]				16 0	305				14 0	230
3		1 75/1		21 0	350		1 75/1		14 0	310
4	Clot	1 75/1		17 5	410	Clot	1 75/1		20 0	410
5	Neg	1 75/1		25 5	260	Neg	[illegible]625/1		28 0	265
6		1 75/1	0 12	22 0	263		1 75/1	0 12	30 0	215
7	Neg	1 75/1	0 12	36 0	125	Neg	1 75/1	0 14	34 0	1 0
8	Neg	1 75/1	0 12	60 0	065	Neg	1 75/1	0 12	68 5	010
9						30 m	Post mortem			
10						Neg	1 75/1	>0 16	>24 hrs	0 000
11										
12										
	Death—8th day					Death—9th day				

TABLE IX B

P d D y N mb	DOG No 91: C l y T t	DOG No 91: L R y T PHR	DOG No 91: All Test mg P m	DOG No 91: Cl g T m m	DOG No 91: Pl l 10^3 mm	DOG No 3: C l y T t	DOG No 3: L R y T PHR	DOG No 3: All T mg P m	DOG No 3: Cl g T m t	DOG No 3: Pl l 10^3 mm	DOG No 35: C l y T t	DOG No 35: L R y T PHR	DOG No 35: All T mg P m	DOG No 35: Cl t g T m m	DOG No 35: Pl l 10^3 mm
		1 5/1		21 0	350	P s	1 75/1		16 0	40		1 75/1		1 0	150
C 1	Neg	1 75/1	0 10	1 0	60	Neg	1 75/1	0 10	14 0	2 0	Neg	1 75/1	0 12	10 0	00
		1 75/1		18 0	3 0	Neg	1 75/1		14 5	250				14 0	315
1				19 0		Neg								17 0	
				15 0	345				16 0					19 0	2 0
3		1 75/1		14 5	255				13 0	390		1 75/1		14 0	360
4	Clot	1 75/1		17 0	10		1 75/1		16 0	385	Neg	1 75/1		0 0	345
5	Pos	6 5/1		21 0	215	Quest	1 75/1		17 0	3 0		1 75/1		14 0	300
6		1 75/1	0 12	7 0	70		1 75/1		19 0	340	Clot	1 75/1	0 14	8 5	335
7	Clot	1 75/1	0 1	48 0	120	Clot	1 75/1	0 10	33 0	05		1 75/1	0 14	44 0	200
8	Neg	1 75/1	0 12	8 0	115		1 75/1	0 1	54 0	155	Pos (?)	1 75/1	0 1	37 0	090
9	Neg	1 75/1	0 1?	58 0	045	Neg	1 75/1	0 1	17 0	110	Pos (?)	1 75/1		83 0	050
10	Clot	1 75/1	0 14	86 5	0 0	Neg	1 5/1	0 10	59 5	0 5	Neg	1 75/1	0 14	113 0	006
11	Neg	1 75/1	0 16	84 0	0 000	Neg	1 75/1	0 16	1 00	010	Neg	1 75/1	0 14	173 0	0 000
12	Neg	1 75/1	>0 18	>9 hrs	0 000	Neg	1 75/1	>0 18	>9 hrs	0 000					
13	Neg			>9 hrs	0 001	Neg	1 75/1	>0 18	>9 hrs	0 000					
	Death—13th day					Death—12th day					Death—1 th day				

P d D y N mb	DOG No 106: C l y T t	DOG No 106: L R y T PHR	DOG No 106: All Test mg P m	DOG No 106: Cl g T m m	DOG No 106: Pl l 10^3 mm	DOG No 108: C l y T t	DOG No 108: L R y T PHR	DOG No 108: All T mg P m	DOG No 108: Cl g T m t	DOG No 108: Pl l 10^3 mm
		1 75/1		15 0	190		1 75/1		17 0	4 0
Co 1	Neg	1 75/1	0 1?	10 0	00	Neg	1 5/1	0 10	10 0	300
		1 75/1		13 0	390		1 75/1		18 5	300
1										
2				12 5	20				16 0	395
3		1 75/1		14 5	310		1 75/1		16 5	375
4	Neg	1 75/1		21 0	305	Neg	1 75/1		21 0	300
5		1 75/1		22 0	250		1 75/1		7 0	70
6	Neg	1 75/1	0 12	35 0	135	Neg	1 75/1	0 14	6 0	05
7		1 75/1	0 14	48 5	125		1 75/1	0 14	40 0	130
8	Neg	1 75/1	0 14	58 0	090	Neg	1 75/1	0 16	55 0	055
9	Neg	1 75/1	0 16	101 0	030	Neg	1 75/1	0 16	>8 hrs	003
10	Neg	1 75/1	>0 16	>6 hrs	001	Q est	1 75/1	>0 16	>6 hrs	0 000
11										
12										
13										
	Death—10 h day					Death—10th day				

TABLES IX A AND B

Tabulation of results with Conley's LeRoy's and Allen's Tests whole blood clotting times and platelets in a group of 10 dogs after 600 r of whole body x ray

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TABLE X

Incidence of Results of Various Tests for Presence of an Anticoagulant Pre-and Post irradiation

	Conley's Test				LeRoy's Test PH Ratio		Allen's Test mg Protamine Sulfate						
	Pos	Quest	Neg	Clot	1 75/1	Other	0 10	0 12	0 14	0 16	>0 16	0 18	>0 18
Pre irradiation	1	0	11	–	21	0	7	6	4	–	–	–	–
Post irradiation	2	5	45	12	65	2 (2 625/1)	7	20	13	5	3	0	3

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The sensitivity of the foregoing tests to added heparin was determined in the dogs prior to irradiation If an anticoagulant were found we were prepared to exsanguinate the dogs and to attempt extraction and identification The results are summarized in Tables IX A IX B and X The studies show that strong anticoagulants of the second phase type were not present There was no evidence for heparin like substances These animals received no therapy In addition repeated *in vitro* titrations of whole blood with toluidine blue and protamine sulfate when done with extreme care in mixing to avoid bubbles and in silicon tubes showed only the anticoagulant action of these drugs thus suggesting that the reduction in clotting times previously reported by us (2) was due to a technical mistake However the occasional reduction in clotting time by the intravenous administration of toluidine blue originally reported by Allen *et al* (13 14) and confirmed by us is yet to be explained satisfactorily Moreover the intravenous toluidine blue did not correct the impaired utilization of prothrombin (26)

On studying the results in Tables IX A IX B and X it is seen that in dog #84 Conley s test was positive on the twelfth day On the same day Allen s test was in the normal range for our laboratory The whole blood clotting time was longer than six hours and no platelets were seen in the peripheral blood Conley s test was definitely positive in one other dog on one day during the postirradiation period It was also positive on one occasion in one dog prior to irradiation

LeRoy s test for heparin was essentially negative throughout the entire period

Allen s test was abnormal on quite a few occasions but could not be correlated either with a positive Conley s test for an anticoagulant or with LeRoy s test An explanation of the abnormal Allen test is not available

I don t think it is necessary to take the time to go through all the observations in each of the dogs These data have been published (22) We came to the conclusion that there was scanty if any evidence for a strong second phase anticoagulant circulating in the blood

Table X summarizes the observations

Alexander Dr Cronkite may I ask what the tests are specifically?

Cronkite In Conley s test a platelet free plasma is prepared with silicon technique in the cold from an animal that perhaps has a circulating anticoagulant This is added in varying concentrations to whole blood from a normal dog and the clotting time is deter

mined at 37 C in glass tubes This detects one microgram per milliliter of heparin

LeRoy s test is a protamine titration of heparin in oxalated plasma Varying amounts of protamine are added An optimal amount of thromboplastin is used and then the system is recalcified The ratio comes out 1 75 of protamine to 1 milligram of heparin

In Allen s test the blood is heparinized Perhaps he should describe it himself

Allen The basic consideration was that a small amount of standard heparin can be added to normal blood without prolonging the clotting time but a point is reached where just a slight increment of heparin will begin to prolong the clotting time We thought that if there was an increase of heparin in the blood that is an endogenous increase of heparin that would reduce the tolerance of blood for exogenous heparin and we should be able to detect the summation effect when we could not detect heparin alone To do that we back titrated We made the blood incoagulable with 91 micrograms of heparin per milliliter of blood and then put each milliliter of blood into a test tube containing increasing concentrations of protamine sulfate ranging from 20 micrograms to 200 micrograms We found that in our setup 120 micrograms of protamine would cause the blood to clot within one hour That is the basis of the test It is quite insensitive It was purposely made that way

Alexander As I understand it it is a measurement of the amount of protamine required to bring back the coagulation time to a fixed point namely one hour of blood to which a fixed amount of heparin has been added

Allen That is correct

Cronkite As a result of subsequent discussions with Dr Tocantins and others we feel that the problem of a first phase anticoagulant cannot be ruled out by these studies I believe Dr Tocantins will discuss that in a little while

Allen What do you mean by a second phase anticoagulant?

Cronkite An anticoagulant which like heparin inhibits thrombin activity

Allen Does heparin have any influence on the first phase?

Cronkite It probably does I don t know

Tocantins Yes it does

Allen We think it definitely does and that it acts there first It would be better if it were just called an anticoagulant It is a *first* stage as well as a second stage inhibitor

Cronkite I would have to agree that heparin probably can be shown to act in all phases of coagulation whereas some of these other inhibitors presumably act only on the first phase

Allen That would be better

Cronkite I stand corrected in the use of the term

The other thing that we repeated was a matter of straight titration of whole blood and plasma with toluidine blue and protamine sulfate When we did this both in glass and silicon tubes using extreme care in the mixing the only thing we were able to demonstrate repeatedly was a prolongation in the clotting time by the addition of these agents which was certainly different from what we originally saw with the Bikini animals (2) However when one injects solutions of toluidine blue or protamine the clotting time is certainly reduced from time to time rather strikingly This Dr Allen has shown repeatedly in the past But when the clotting time was reduced by the intravenous toluidine blue no improvement was seen in the utilization of prothrombin

Allen We found the same thing

Cronkite We went on to study all the plasma factors we were able to that were concerned with blood coagulation It has been reported by many investigators that the prothrombin level is not significantly altered (1,2 14 15,22 27) Occasionally there is a terminal reduction in the level and in such animals at autopsy there is a diffuse bacterial necrosis of the liver In addition two-stage prothrombin levels were performed with and without the addition of Ac globulin There was no significant difference thus suggesting that adequate amounts of Ac globulin were present

Fibrinogen levels were studied in a group of dogs In general there was a biphasic response (22) Fibrinogen levels began to increase at about the same time that there was clinical evidence of infection An occasional dog developed fibrinogen levels as high as 2 Gm per 100 ml of blood

In the next study the evolution of serum prothrombin conversion accelerator (spca) (28) and the levels of its precursor were studied Normal dogs prior to irradiation demonstrated about 40 per cent enhancement After irradiation when the thrombocytopenia had developed there was a marked diminution in the evolution of spca That this apparent deficiency was not due to a diminution of the precursor was shown by the fact that more than the normal enhancement was obtained when coagulation was forced by agitation or the addition of thrombin thromboplastin or platelets to the freshly

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Allen We think it definitely does and that it acts there first It would be better if it were just called an anticoagulant It is a first stage as well as a second stage inhibitor

method is in order Platelets were counted by the phase contrast method of Brecher and Cronkite (35) By this method the accuracy is the equal of any hemocytometer count The accuracy can be set at any desired level by the use of multiple pipettes The accuracy of any hemocytometer count is determined by the combined errors of pipette field chamber and technician Since the latter is difficult to approximate the following mathematical considerations determine the minimum error possible

$$\text{Standard deviation of mean* in per cent} = \sqrt{\frac{8000}{\#\text{ of cells counted}} + \frac{20}{\#\text{ of pipettes used}} + \frac{20}{\#\text{ of chambers used}}}$$

for the average platelet count

1 pipette and 1 chamber $\sigma = 11\%$
2 pipettes and 2 chambers $\sigma = 8\%$
8 pipettes and 8 chambers $\sigma = 4\%$

From the above statistical considerations it is apparent that the error of any hemocytometer count increases rapidly as the total count decreases because so few cells are counted In addition the use of the dark phase contrast and the 1 per cent ammonium oxalate solution make possible an absolute identification of platelets (35) The appearance of the platelets under various conditions and in the hemocytometer chamber has been described (35) In the chamber the platelets are refractile purplish with distinct granules and one or more processes develop as the platelets settle out Some look like sperm others look like cockleburs The appearance is distinctly different from dust bacteria molds yeast etc

Ferguson It is very important is it not how the blood is collected into the pipette?

Cronkite Oh yes It has to be done with extreme rapidity The pipettes are filled either with blood flowing directly from the vein or from a few drops which are let fall onto a silicon watchglass It has to be done with great rapidity or else the accuracy of the platelet count decreases There will be agglutinated platelets in the hemocytometer

The depression in platelet count is a function of dose between 150 to 400 r Above 400 r the depression becomes maximal and thus independent of dose Generally the platelet counts trend upward for the first three days after exposure With higher doses of radiation the survival time becomes so short (three to four days)

* Based on studies of Berkson J Magath T B and Hurn M *Am J Physiol* **128** 309 *(1940)*

drawn blood * It appears that there was no deficiency of the precursors of spca but that there was an impairment of the conversion of the precursors to the active form presumably resulting from the severe thrombocytopenia However platelets apparently play a passive role in the formation of spca because it could be evolved in large amounts by forcing coagulation in the absence of platelets

The studies of Holden *et al* (15) on hypothromboplastinemia were repeated in part and confirmed However we are inclined to think that the observations of a prolonged clotting time of the highly centrifuged supernatant plasma can be explained on the basis of the thrombocytopenia Much more work must be done in order to establish the existence of a true plasma thromboplastin and to determine it quantitatively The current techniques are unsatisfactory At the present time it appears doubtful that there is a plasma thromboplastin comparable to tissue thromboplastin

The antihemophilic factor activity of irradiated dogs was studied by Penick *et al* (29) in Dr Brinkhous laboratory

Brinkhous I shall comment on this later

Cronkite We desire to study the vascular factors concerned with hemostasis by direct microscopy but have done practically nothing in our own laboratory up to the present time Copley is studying this † Haley and his group (30) at the University of California at Los Angeles have studied the reactivity of vessels and the presence of vasotropic substances in plasma Their evidence points towards the liberation of VDM and VEM at different times after irradiation The relationship of these substances to the bleeding tendency is not clear

Furth Bigelow Ross Kahn *et al* (31 32 33 34) have attempted to explain the hemorrhagic tendency on the basis of endothelial damage Their conclusions are based on the appearance of large numbers of red cells in the thoracic duct lymph beginning six to seven days after irradiation An intact capillary endothelium does not leak The postulate of direct or indirect injury to the endothelium is reasonable and may be related to the thrombopenia Time does not allow a discussion of this work but the references are given for those interested

The bulk of our efforts have been concentrated on the study of the platelet Since many of our conclusions are based on an accurate estimate of the platelet count a statement on the reliability of our

* Jacobs G J and Cronkite E P Serum prothrombin conversion accelerator (spca) after lethal whole body irradiation (Unpublished article)
† Copley A L Personal communications

that the animals die before the depression begins

In general it can be stated that the bleeding tendency increases with the degree of thrombopenia and that bleeding is virtually absent with doses of radiation that do not depress the platelet count well below 100 000 per mm³ For example after 150 r (sublethal and no external bleeding) the platelets fall to about 100 000 per mm³ (Figure 83) After 600 r the typical response is shown in Figure 84 When the platelets start down the decrease is approxi

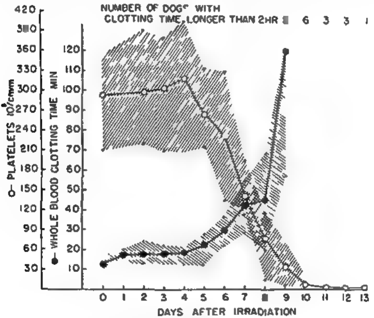

FIGURE 84 Inverse relationship of whole blood clotting time and the platelet count in 9 dogs irradiated simultaneously The mean and range is shown Reprinted by permission from Jackson D P *et al* Further studies on the nature of the hemorrhagic phase of radiation injury *J Lab & Clin Med* 39 449 (195–)

mately linear with time reaching the minimum values of 0 10 000 by the ninth to the eleventh day Figures 85 and 86 show the relationship between the platelet count and the granulocyte count for various mortalities At 10 per cent mortality the platelet depression is marked Only a moderate granulocytopenia occurs Very few animals develop infection

Alexander Dr Cronkite you mentioned the fact that the platelets tend to agglutinate very rapidly which is certainly a normal phenomonenon Have you any evidence that the platelets fail to

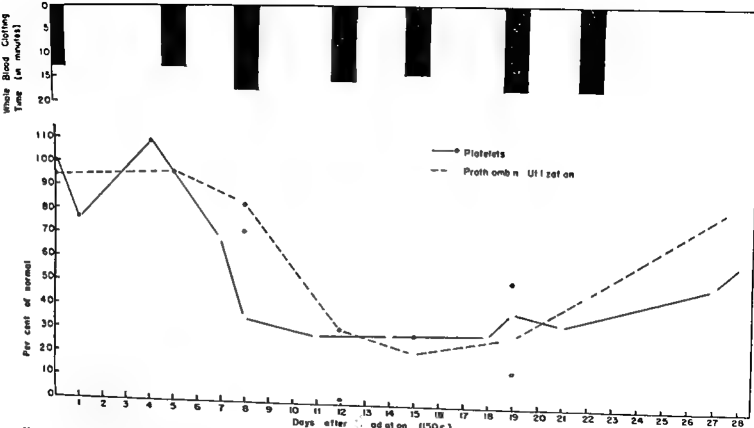

FIGURE 83 The relationship of platelet levels prothrombin utilization and whole blood clotting times in dogs after exposure to 150 r a sublethal dose of 2000 kvp x ray The mean values and range of 8 dogs irradiated simultaneously

agglutinate in the freshly shed blood of these irradiated dogs at any interval before the platelet count drops markedly?

Cronkite We have no good evidence on that That is a subject we should study It is my impression that they do not

Alexander That they do not?

Cronkite I mean that there are no significant differences from normal blood but we have not really studied that accurately

Allen Wouldn t it be fair to say that occasionally a platelet count must be discarded even though it is a thrombocytopenic one because of agglutination?

Cronkite Oh yes

Allen It is much the same as the normal situation

Alexander Well I was curious to know whether there was any retardation in this tendency to agglutinate or any increase in the tendency to agglutinate early in the postirradiation phase

Cronkite We have not studied this sufficiently well to have any firm belief on it It is just an impression that they will agglutinate as easily after irradiation as before As the platelet count goes down the probability of any random collision of platelets is less so that there is less agglutination of course And in this early stage in which the platelet counts occasionally go up to very high levels the probability of agglutinating I think is greater It however has not been put to a critical study nor would I know how to study it critically

Brambel What would you think about Helen Payling Wright s technique?

Cronkite We thought of doing it but we have not

Brambel That work has been confirmed in connection with the anticoagulants in this country

Cronkite It probably would be a good technique to use

Brambel There is another one I can t think of the name of the investigator at the moment

Mann Moolten (36)?

Brambel Yes

Cronkite We just have not tried it We have had no experience with those techniques One thing that worries me about them is that I think they may be a function of the platelet count itself and the probability of a random collision of platelets with each other or with the surface will vary with the count This correction would probably have to be introduced into that type of technique which I don t believe either Moolten or Dr Wright has done

As the mortality of the animals goes up to 80 per cent or 100 per

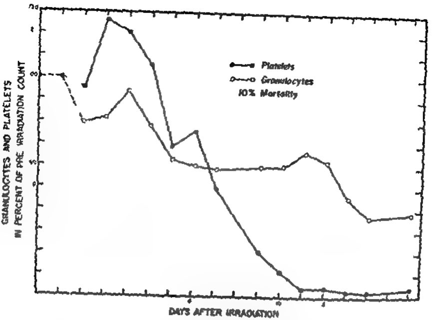

FIGURE 85 The mean platelet count and granulocyte count of 10 dogs that received radiation producing a 10% mortality Note elevated platelet count 2 to 4 days after irradiation and the severe thrombocytopenia but not complete disappearance from the 11th to 16th days and the less severe and slowly developing granulocytopenia

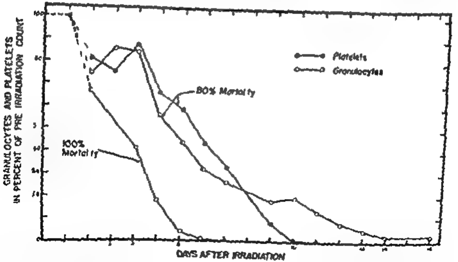

FIGURE 86 The mean platelet and granulocyte count in 10 dogs receiving a dose of radiation resulting in 80% and 100% mortality Platelets completely disappear by the 10th day Pattern of platelets is then the same at 80% and 100% mortality At 80% granulocytopenia is less severe and develops much less rapidly

cultured? Quite often the culture of bone marrow is found to be positive when the blood is negative That is true in typhoid fever malaria and a number of other diseases

Cronkite We have cultured spleen liver bone marrow heart blood and so on occasionally at autopsy

Tocantins I mean *in vivo*

Cronkite The only one that is frequently positive in the dog is the liver but this apparently happens in the normal dog

Wright Dr Cronkite couldn t the fever be in part due to the hemorrhage?

Cronkite Oh certainly

Wright In your comments you apparently considered fever as evidence of infection

Cronkite It could be one or the other or a combination of both

Best Did you try antibiotics?

Cronkite I would rather not go into the antibiotic problem in radiation injury It would take too much time and in respect to dogs I am on one side of the fence and none of the other people concerned are here to state their side of the problem I will just say this We have not had any success with aureomycin streptomycin or chloromycetin in dogs One dog has survived what was believed to be a supralethal dose of x ray after receiving huge doses of penicillin and intermittent transfusions

Figure 87 demonstrates the changes in the platelets in a group after 400 r There is no difference in the platelet response in survivors and nonsurvivors among animals that were treated and not treated The treatment in this case consisted of penicillin and whole blood transfusions

In general it has been our experience that the bleeding tendency increases with the degree and the length of the thrombopenia and that bleeding is not seen until the doses of irradiation are sufficiently high so as to depress the platelet count well below 100 000 per cubic millimeter For example after 150 r which is a sublethal dose of radiation no external bleeding is seen and at autopsy there is little evidence of iron in the lymph nodes to suggest there had been hemorrhage during the course of the injury

I wish to emphasize that the entire picture in the blood may be significantly altered by shielding the bone marrow or by unequal depth doses There may be a completely different picture if the entire dose of radiation is given only to one side of the animal or if the head is shielded as Dr Allen has done or if other portions of the body are shielded

cent the response of the platelets is uniform There is apparently less tendency at these higher mortalities and higher doses of irradiation to have the initial increase in the platelet count The platelet count uniformly hits zero around the tenth day By zero is meant less than 5000 platelets In many animals no platelets are found At 80 per cent mortality the granulocyte depression as can be seen in Figure 86 is much greater than it was at the 10 per cent mortality And when 100 per cent mortality is obtained granulocytes have virtually disappeared by the sixth or seventh day Infections are suggested by an increase in the rectal temperature of these animals by the eighth day on

Allen There is a curious thing about that If their blood is cultured a septicemia is detected only occasionally I am convinced just as you are that there is infection yet it does not seem to enter the bloodstream and stay there with any degree of constancy

Cronkite There is infection but there is not obvious bacteremia until the very terminal stages We have on occasion cultured the edema fluid from the cellulitis that develops and that is positive But in the same animal with virtually no granulocytes the blood cultures will be negative Everybody has apparently had the same experience

Best At what stage is the liver necrosis found?

Cronkite It is a terminal thing In this respect Dr Mann was telling me his father had cultured dog liver many years ago and found the saprophytic anaerobes apparently these commence to multiply in terminal stages In the liver multiple small foci of necrosis may develop

Best That is the picture one gets after tying off the hepatic artery

Cronkite Yes the same type of picture

Flynn In very few bacterial infections do the organisms multiply within the bloodstream In most cases the bacteria are removed by the reticuloendothelial system almost as soon as they enter the blood even in the presence of a granulocytopenia Thus a positive blood culture means that the organisms have been intercepted in transport Assuming the same facility of culturing the probability of random interception of the organisms by removing a small fraction of the total blood will obviously vary with the number of organisms present the intermittant or periodic frequency of their transport the size of the blood sample taken and the number of samples cultured

Tocantins Has the bone marrow of these animals ever been

As a result of these studies on the relationship of dose to changes in the levels of platelets and the tendency to bleed paralleling the thrombocytopenia more studies on the relationship of the platelet count to the bleeding tendency were performed

Another group of animals were given 600 r Platelet counts went down to very low levels by the ninth day (see Figure 88) From

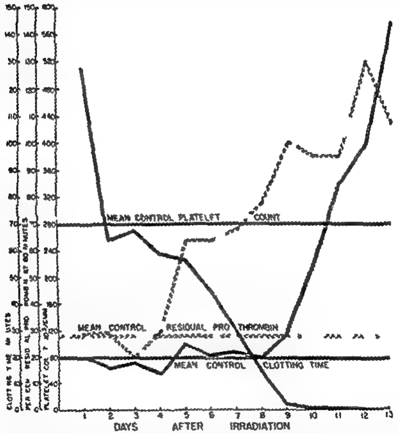

FIGURE 88 Platelet counts blood clotting times and residual prothrombin after 60 minutes of clotting in dogs after 600 r whole body irradiation Reprinted by permission from Jackson D P et al Prothrombin utilization in radiation injury Am J Physiol 169 .09 (1952)

the tenth day on platelets were virtually absent Residual prothrombin progressively increased inversely with the platelet count Whole blood clotting times started up on the ninth day after the platelet count was down to virtually zero and the residual pro

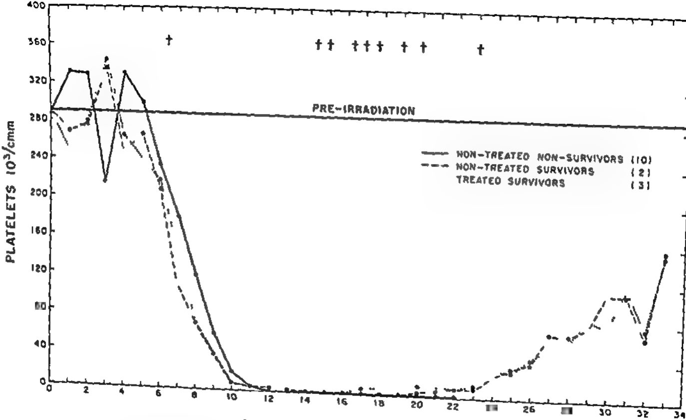

FIGURE 57 Platelet counts in dogs after 400 r (LD_{50}) that survived with and without treatment by administration of penicillin and blood transfusions and in dogs that died

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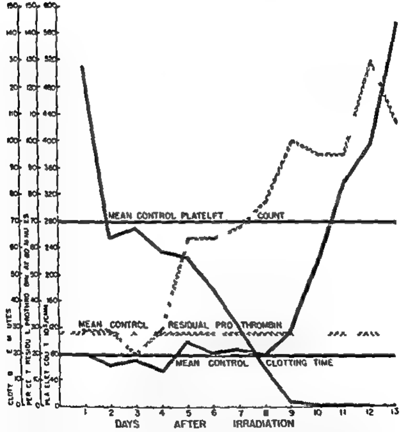

FIGURE 88 Platelet counts blood clotting times and residual prothrombin after 60 minutes of clotting in dogs after 600 r whole body irradiation Reprinted by permission from Jackson D P et al. Prothrombin utilization in radiation injury *Am J Physiol* 169 208 (1952)

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thrombin was close to 100 per cent of the normal prothrombin The clotting times increased in excess of two hours, and some of the samples of blood were incoagulable in the terminal stages

In Figure 89 the residual prothrombin is shown in four groups prior to irradiation 1 to 4 days after irradiation 5 to 8 days after

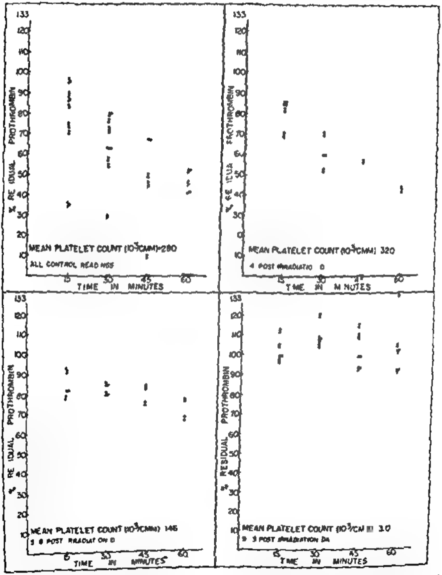

FIGURE 89 Residual prothrombin scatter diagrams for an hour after withdrawal of blood for the pre irradiation control period 1 to 4 days 5 to 8 days and 9 to 13 days after irradiation Reprinted by permission from Jackson D P et al Prothrombin utilization in radiation injury *Am J Physiol* 169 208 (1952)

irradiation and 9 to 13 days after irradiation The progressive decrease in the consumption of prothrombin is apparent During the time interval 9 to 13 days after exposure virtually no prothrombin is consumed over a 2 hour period at 37 C In the above studies the method of Buckwalter *et al* (37) was used to measure residual prothrombin

Alexander These are scatter diagrams Dr Cronkite?

Cronkite This is the maximum and minimum of all the observations made on a given day The points in Figure 89 represent the residual prothrombin after blood was drawn into glass tubes

Alexander The question I should like to put is whether let's say on the third fourth and fifth postirradiation days you have isolated or multiple observations where the platelet count is relatively normal but residual serum prothrombin is distinctly high? The reason I ask is to learn whether there is any evidence of a qualitative change in the circulating platelets and not necessarily a change in their number

Cronkite We are going to come to that point a little bit later but not in respect to what happens in the first few days We have not seen anything different

There is another thing that I forgot to mention earlier and which I think is important After the fourth day there are few if any megakaryocytes in the bone marrow and I think it is probably fair to assume that platelet production has stopped Hence the age distribution of platelets *per se* is continually shifting to the older side as the platelet count goes down The same thing occurs in the recovery phase the age distribution of platelets is shifted to the left No critical studies on the influence of platelet age on platelet function have been performed by us as yet We have just been thinking about the functional differences between young and old platelets senile platelets

This good correlation between platelet count prothrombin utilization etc was confirmed in another group of three dogs as shown in Table XI Platelets decreased in number residual prothrombin increased clotting times became very prolonged white blood cells fell to virtually zero and hematocrits decreased to low levels certifying that the dogs were bleeding not producing red cells and perhaps destroying cells

Lewis There appears to be a day or two overlap at the fourth or fifth day where the platelets are still normal but the prothrombin consumption has changed Is this a consistent finding?

Cronkite It is very hard to give a precise relationship here on

TABLE XI

Repetition of Study on Relation of Platelets, Clotting Times, and Prothrombin Utilization in 3 Additional Dogs Exposed to 600 r

Post X ray Day	Hematocrit %	WBC per cmm	Platelets per cmm	Clotting time min	Residual prothrombin at 60 minutes in %
Control mean of 3 days	46 9	10 300	260 000	19 0	28
1	46 7	10 900	510 000	20 0	30
2	46 5	5 200	255 000	16 0	29
3	44 0	11 100	277 000	19 0	20
4	47 5	6 200	238 000	14 0	30
5	44 5	2 000	223 000	25 0	63
6	43 7	1 500	180 000	21 0	63
7	43 5	700	122 000	23 0	69
8	38 0	600	60 000	20 0	78
9	36 7	700	10 000	28 0	101
10	35 5	600	3 300	54 0	96
11	30 7	200	800	85 0	96
12	27 7	200	500	99 0	131
13	25 0	100	500	144 0	107

that In general the prothrombin utilization diminishes as the platelets go below 200 000 It is not possible to have the precision one would like One sees that there is that general trend Occasionally, it does look as if the utilization is impaired before the platelets are significantly below the normal pre irradiation level At which time the age distribution shows older platelets

Flynn Do you think that hyperplateletemia on the first day of Table XI could be a result of the impact of the radiation on the megakaryocytes and their subsequent fragmentation?

Cronkite I don t know what the cause is

Allen The leukocytes also go up that first day

Cronkite There is variability in the leukocytes in our experience

Flynn According to Table XI the elevation of leukocytes is not as striking as the platelet elevation

Cronkite Sometimes they go up and sometimes they don t I am not willing to say with any certainty what happens to the leukocytes during the first forty eight hours It is quite uniform to have this increase in the platelet level sometimes prolonged for three

or four days Once it starts to go down there is a linear regression with time

In Figure 83 is shown what happens to the platelet count, the utilization of prothrombin and the whole blood clotting time in a group of dogs after 150 r With this dose of irradiation the dogs did not bleed there was no prolongation in the clotting time and there was no mortality The rough correlation of the defect in utilization of prothrombin with the decrease in the platelet level is apparent.

I won t take more time on this I know Dr Tocantins is going to have something additional to say about it

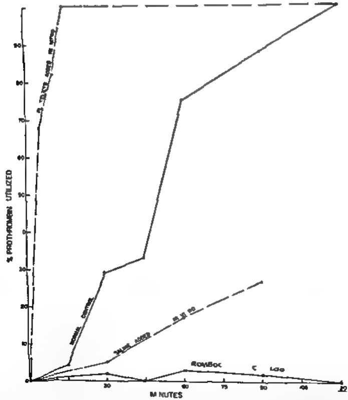

FIGURE 90 Mean prothrombin utilization in per cent for a group of 4 dogs before irradiation (normal control) 8 to 1_ days after irradiation (thrombocytopenic blood) thrombocytopenic blood diluted with saline and thrombocytopenic blood with platelets added to give a resulting concentration of 8000 per mm Reprinted by permission from Cronkite E P *et al* The hemorrhagic phase of the acute radiation syndrome due to exposure of the whole body to penetrating ionizing radiation *Am J Roentgenol* 67 797 (195?)

TABLE XI

Repetition of Study on Relation of Platelets, Clotting Times, and Prothrombin Utilization in 3 Additional Dogs Exposed to 600 r

Post X ray Day	Hematocrit %	WBC per cmm	Platelets per cmm	Clotting time min	Residual prothrombin at 60 minutes in %
Control mean of 3 days	46 9	10 300	280 000	19 0	28
1	46 7	10 900	510 000	20 0	30
2	46 5	5 200	255 000	16 0	29
3	44 0	11 100	277 000	19 0	20
4	47 5	6 200	238 000	14 0	30
5	44 5	2 000	223 000	25 0	63
6	43 7	1 500	180 000	21 0	63
7	43 5	700	122 000	23 0	69
8	38 0	600	60 000	20 0	78
9	36 7	700	10 000	28 0	101
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Cronkite Sometimes they go up and sometimes they don t I am not willing to say with any certainty what happens to the leukocytes during the first forty eight hours It is quite uniform to have this increase in the platelet level sometimes prolonged for three

creased the consumption of prothrombin About the latter Dr Tocantins will have more to say In Figure 91 is a quantitative estimation of the influence of increasing amounts of separated platelets on prothrombin utilization at five ten and fifteen minutes after withdrawal of blood The remarkably rapid rate of consumption of prothrombin by thrombocytopenic blood after the addition of a few platelets is apparent

Alexander You have never added platelets from an irradiated dog to thrombocytopenic plasma?

Cronkite No we have not done that because in the early phase of radiation injury it did not occur to us and so few animals have survived that there just were not enough animals available to act as donors in the recovery phase

An example of the relation of platelet levels to prothrombin utilization in the recovery phase is seen in Figure 92 About the twenty third day or so the platelet count starts up The count drops in two to three days and then there is a steady increase to the normal range over a period of about two weeks However as the

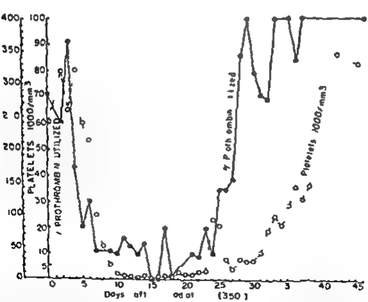

FIGURE 92 The relation of the prothrombin utilization and platelet count in a dog that survived 350 r total body x ray Reprinted by permission from Cronkite E P et al The hemorrhagic phase of the acute radiation syndrome due to exposure of the whole body to penetrating ionizing radiation *Am J Roentgenol* 67 797 (1952)

With these correlations — and that is all they were just a relationship of one thing changing as another did — we wondered whether these defects could be corrected by the *in vitro* addition of washed platelets Platelets were prepared in different manners It made no difference whether platelets were from our sequestrene treated plasma or from citrated plasma and washed In Figure 90 is shown the correction of impaired prothrombin utilization by the addition of separated platelets to the whole blood Over a two hour period *no prothrombin was consumed by the thrombocytopenic blood* but the addition of 8000 platelets per mm³ strikingly accelerated coagulation and prothrombin consumption Dilution with saline also in

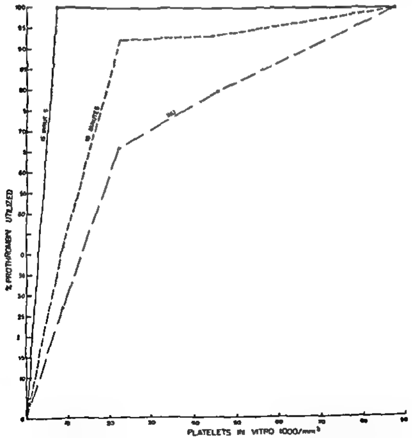

FIGURE 91 Influence of titration of the thrombocytopenic blood with varying amounts of platelets upon prothrombin utilization Reprinted by permission, from Cronkite E P *et al* The hemorrhagic phase of the acute radiation syndrome due to exposure of the whole body to penetrating ionizing radiation *Am J Roentgenol* 67 -97 (1952)

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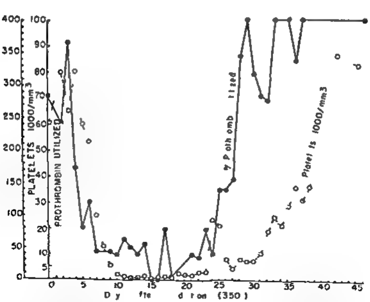

FIGURE 92 The relation of the prothrombin utilization and platelet count in a dog that survived 350 r total body x ray Reprinted by permission from Cronkite E P et al The hemorrhagic phase of the acute radiation syndrome due to exposure of the whole body to penetrating ionizing radiation *Am J Roentgenol* 67 797 (1952)

platelet count starts up the utilization of prothrombin rapidly returns to and stays in the normal range even when platelets are less than 100 000 per mm³ These platelets are largely newly formed Whether the newly formed platelet is more effective than the "senile platelet remains unanswered The above relation has been seen in each dog that has survived doses of x ray greater than the mid lethal dose

Allen When does the platelet count begin to go substantially up and to stay up?

Cronkite After twenty seven to twenty eight days the sustained continued rise occurs

Allen It would certainly look as though you were having a different type of platelet wouldn t it?

Cronkite Yes these are newly formed platelets The pattern has been seen in each surviving dog we have studied

Allen What was your exposure dosage here?

Cronkite In this case it was 350 r The animal survived We have observed the same thing after 400 and 600 r *

Seegers If I understand Figure 92 correctly there is 100 per cent prothrombin utilization when the animal is recovering but when the platelet count is still low

Cronkite Yes 70 to 100 per cent of prothrombin was utilized at sixty minutes when platelet levels were below 100 000 per mm³, however the age distribution of platelets was different If that means anything I don t know

Best Were you able to do bone marrow studies just at that crucial time?

Cronkite On this dog no On other animals we have done bone marrow studies Unfortunately all of the animals died No megakaryocytes were present after six days

Best Just as they begin to go up did you have any observations at that time?

Cronkite That was done once

Best Not with the platelets?

Cronkite Megakaryocytes were not present in the sample That does not mean they were not there There is a big sampling error in aspirated marrow There were other signs of activity in the bone marrow such as mitoses myelocytes and normoblasts It was just enough to make one wonder whether megakaryocytes really make platelets

* The one survivor from 600 r that was treated with penicillin and blood transfusions

Best That is what I was trying to get at

Cronkite I shall not go into that further We expected to see megakaryocytes but did not in this dog that survived

Flynn But could you say the increased prothrombin utilization occurred at the time when there was renewed bone marrow activity? Is that the conclusion?

Cronkite Yes there was renewed bone marrow activity but in this sample there were no megakaryocytes

Warner With that essentially normal prothrombin utilization at the time when the platelets were still quite low that is the amount utilized in sixty minutes did you have a curve of utilization?

Cronkite Yes I don t have a slide of it It was essentially normal

Tocantins There is one other feature about these platelets that might be brought out now I understand that most of the platelets appearing at this stage are larger than normal Their volume may be as much as five to eight times the normal if the pattern of platelet recovery after irradiation is anything like the pattern of platelet regeneration in thrombocytopenia produced by antiplatelet serum The length of the platelets may also be great to the point where there may be confusion sometimes as to whether one is seeing platelets or red blood cells in the counting chamber since some of the platelets come fully as large as red blood cells Although their number may be small they make up for it by their volume

Flynn Do they have the same staining characteristics the same morphology?

Tocantins Not quite the same morphology They take of course the same type of stain but the granules are much coarser and deeper stained The granules fill the entire platelet all the way out to the periphery instead of being bundled up in the center as in normal platelets

Cronkite The platelets are much larger in the Wright stained smear and hyperchromatic Careful morphologic studies on the platelets with phase microscopy are contemplated but they have not been performed as yet in the recovery phase

Since the *in vitro* studies on platelets were so rewarding and because Lawrence and Valentine (38) had observed that cross circulation of a normal cat with an irradiated cat restored the platelet level temporarily and that the bleeding tendency was directly related to the platelet level we decided to try platelet transfusions The latter had been made possible with the development of a simple method for the separation and concentration of platelets by Dillard *et al* (39) The method depends upon the use

of di sodium salt of ethylene diamine tetra acetic acid (sequestrene) which removes divalent cations and may interfere with coagulation in other as yet undertermined ways Platelet suspensions in plasma are obtained with minimal agglutination and virtually no morphologic changes appear over a period of hours The yield of platelets is roughly 40 to 60 per cent of those that were present in the whole blood However these platelets if more than a few hours old will not circulate upon injection into the normal or the irradiated animal

Daily platelet transfusions were first tried in guinea pigs using cardiac punctures Higher levels of platelets were maintained in the transfused irradiated (400 r) guinea pigs than in the control guinea pigs The results were not very impressive Quite a few of the animals died from cardiac tamponade Subjectively it appeared as if there were less bleeding Due to technical difficulties the guinea pig work was dropped and efforts shifted to the dog This necessitated a blood donor colony of large dogs and the use of small recipients The immediate aim was to transfuse daily a volume of platelets equal to the number normally present in the recipient

The results on the first dog (#335) that was transfused are shown in Table XII Platelet transfusions were commenced on the eleventh day after irradiation with 400 r Four hundred r was used because survival time is a little longer and would permit more time during which the dog could be studied

It is apparent that the yield of platelets was variable and that the platelets do not distribute uniformly with the time after injection On the fourteenth day (the fourth day of transfusions) the platelet count was up to 190 000 and the prothrombin utilization had increased to 66 per cent At this point curiosity demanded that the animal be sacrificed along with a control dog that was receiving platelet free plasma in order to compare the relative degrees of bleeding in the two animals

Before continuing a comment on the mixing time of platelets is appropriate Apparently separated transfused platelets do not distribute homogeneously throughout the body as might have been anticipated If serial counts are performed at regular intervals considerable variation in the count is obtained There is it would seem not a uniform mixing and disappearance time as is seen with red cells and electrolytes However in rough terms the maximum levels attained were approximately 50 per cent of the calculated values

Alexander Dr Cronkite was the blood from one donor?

Cronkite No from different donors

Alexander One recipient received platelets from different donors?

TABLE XII

Example of Effect of Platelet Transfusions in Two Thrombopenic Irradiated Dogs (400 r)

	Days after Irradiation	No of platelets injected × 10	Platelet count × 10			% prothrombin utilized in clotting for 60 minutes
			before injection	10–30 min after injection	_ hrs after injection	
Dog #335	11	18	8	20	95 000	15
	12	13	40	140	125 000	10
	13	17	58	125		27
	14	25	115	190		66
Dog #336	5	9	295	255		100
	6	20	190	275		47
	7	14	165	225		41
	8	34	175	205		100
	9	30	125	200		77
	10	4	130	120		60
	11	39	53	130		29
	12	114	30	147		1
	13	385	70	185		37
	14	78	90	235		61

Cronkite Yes There was a total of eight different donors Two dogs would be used every other day The total platelets came from eight different animals

Alexander One is not justified in concluding that on the fourteenth post x ray day this dog received younger platelets?

Cronkite Oh no I don't think so The platelets given at this time were from animals that had been bled on the first day

Alexander Then they might have been younger

Cronkite In subsequent experiments probably they were somewhat younger About 25 per cent of the blood volume was withdrawn from each donor in order to get enough blood

Alexander That of course also might be a variable in estimating the longevity of the circulating platelets in the recipient dog

Cronkite That is true

Flynn By this irregular distribution Dr Cronkite do you mean that if you did a count say at eight o clock you got one value and then if you did a count at nine o clock it might vary by 50 or 60 per cent?

Cronkite It varied considerably but usually not that much We have not done enough of it to try to work out any pattern except that so far there has not been as we had hoped there might be a uniform mixing and disappearance rate It has been erratic

The relationship between platelet levels and prothrombin utilization was not nearly as consistent with the transfused platelets as it was in the degenerative stage (Figure 84) Virtually no prothrombin was utilized until the platelets were transfused up to 190 000 per mm³ No explanation is readily available for this discrepancy The investigations so far have only scratched the surface of the behavior of transfused platelets It is apparent however that transfused platelets do not behave as one might have prophesied from *in vitro* studies

A study of the gross appearance of the lymph nodes from the platelet transfused animal (#336) and its control showed that all of the lymph nodes in the control animal were hemorrhagic and that only one lymph node from the animal receiving platelet transfusions appeared as if there may have been some bleeding into it Microscopically there were a few red cells in the peripheral sinus The type of bleeding into the peripheral sinus extends into the medullary sinuses in the lymph nodes of the nontransfused dog All lymph nodes are loaded with red cells

Tocantins We saw the same sort of thing in animals that had received antiplatelet serum and had been made thrombocytopenic I don t know whether I am misconstruing what you just said but we interpreted this as meaning that the cortical sinuses of the lymph nodes were draining blood that had been extravasated into the neighboring tissues In our animals the peritoneal lymphatics were loaded with extravasated blood and we saw the same thing in the lymphatics around the bronchi and in the lungs

Cronkite That is in part an explanation for it but on serial sacrifice of the dogs one sees bleeding into the lymph nodes in areas in which there is no visible bleeding distal to them so it has been concluded that there is also actual hemorrhage into the sinuses of the lymph nodes Furth *et al* (31 32 33) are inclined towards your explanation

Tocantins Sometimes when most of the hemorrhage was in the abdomen the abdominal lymph nodes were grossly streaked with red stains

Cronkite In Table XII are also shown results on dog #336 the second dog that was transfused with platelets In dog #336 transfusions were commenced on the fifth day Again there was variation

TABLE XIII

Platelet transfusions in dogs Platelet counts $\times 10^3/mm^3$

Dog #		Days after irradiation 0	5	6	7	8	9	10	11	12	13	
346	Before transfusion	320	395	275	135	210	120	110	105	95	32	(died)
	After transfusion			225*	260	160*	165	155	145	145	200	
347	Before transfusion	300	270	295	190	275	235	160	150	125	(sacrificed)	
	After transfusion			385	395	375	345	245	290	275		
349	Control	560	435	335	190	95	43	8	7	3	(died)	

Day old platelets

in the platelet yield and the elevation in count after transfusion After a couple of bad days in harvesting platelets the donor volume of blood was quadrupled (day 11 through 14) and the platelet levels brought up progressively to 235 000 per mm^3 The prothrombin utilization varied in an erratic manner but tended to follow the platelet levels Again curiosity ruled and the dogs were sacrificed for histologic study The difference was striking both grossly and histologically In the control animal that received platelet free plasma all lymph nodes were diffusely hemorrhagic There was no bleeding grossly or microscopically in the tissues of the platelet transfused dog

In repeating these studies 600 r was chosen as the dose of radiation because survival rate is nil and hematopoiesis was known to be eradicated for a period of at least three weeks which is the longest survival time of an untreated 600 r dog Three dogs were irradiated simultaneously two received platelet transfusions (#346 and 347) and one (#349) received platelet free plasma The results are tabulated in Table XIII In dog #346 there was not an increase in platelet levels on the sixth and eighth days when day old platelets were used * In dog #347 platelets increased after each transfusion Again prothrombin utilization was erratic only roughly following the platelet levels However there was no bleeding in either of the transfused dogs and there was extensive hemorrhage into the tissues of the control dog #349 both grossly and microscopically

The type of bleeding demonstrated in the thrombocytopenic state appears to be histologically different from the bleeding seen in the dicumarolized animal with its pure clotting defect The distribution of the bleeding into the lymph node in a dog that received 300 mg of dicumarol for 10 days is less regular and is spotty in appearance †

Brinkhous Isn t there a greater loss of lymphocytes in your animals treated with 600 r than in the 300 r animals?

Another thing about radiation that cannot be taken up now is the matter of the species difference in regeneration The dog at 600 r shows only minimal regeneration of lymph nodes through the twentieth day Other species show extensive regeneration 7 to 10 days after irradiation

Cronkite Oh yes

* Subsequent studies have shown that day old platelets circulate poorly in normal and irradiated dogs and occasionally induce a thrombocytopenia

† Subsequent studies have shown that the cortical sinuses of the lymph nodes of dicumarolized dog may be filled with blood when there is extensive hemorrhage distal to the lymph node

Allen I should like to point out that I have for some time raised the question as to whether this is a hemorrhage in lymph nodes or whether it is that so to speak the flood gates to the lymphatics open up and there is really one circulation instead of two With the distribution of hemorrhage being so different with dicumarol and the other being limited to the cortical sinuses largely it raises the question in my mind as to whether this is not pretty good evidence that one is hemorrhage and one is perhaps a filtering out system of a lymphatic drainage Furth (33) who has been working on this down at Oak Ridge has recently gotten some interesting data He has been studying the drainage of the lymph ducts particularly the thoracic duct and has raised that question

Cronkite Are you postulating a shunt between the vascular bed and the lymph bed in the lymph node that could open up and let blood out into the lymph bed?

Allen I am wondering very seriously whether to some extent that does not occur It is a matter of interpretation

Cronkite Certainly when this occurs the thoracic duct lymph is grossly bloody

Allen Yes it is

Cronkite The thought of a shunt never occurred to me

Allen The nodes that are most hemorrhagic are as Dr Tocantins has said those draining the largest vascular areas There are hemorrhagic lymph nodes in the neck it is true or what we have been calling hemorrhagic when in reality they are engorged But the most strikingly hemorrhagic are those along the portal system

Cronkite The mesenteric lymph nodes certainly are hemorrhagic

Allen Yes

Cronkite Around the cecum and the pylorus of the dog the lymph nodes are almost 100 per cent hemorrhagic Occasionally the retropharyngeal lymph nodes will not be hemorrhagic but axillary and inguinal are 100 per cent hemorrhagic in our experience The hemorrhagic appearance occurs earlier in the mesenteric lymph nodes I think that is true but the concept of shunt I have never heard before

Allen I dont want to claim originality for it though we have thought of it many times We have not actually approached it experimentally in the manner Furth has done

Cronkite Be that as it may if there is some way that the vascular bed can open into the lymph vessels in the lymph nodes this can be prevented by platelets

Allen That is right

Cronkite And I think it brings up the point that in all hemorrhagic disorders the critical thing is really the vascular bed Perhaps these other things, which are so interesting to measure and study are of secondary importance

Best Can you prevent this opening up of the flood gates or whatever you call them?

Cronkite With dicumarol?

Best No Can you prevent it by platelets when they do not prevent hemorrhage in other places?

Cronkite Yes with the transfused platelets as inadequate as they are there is no bleeding anywhere

Best Does this type of bleeding react to platelets before the other bleeding in any other tissues?

Wright Do you mean dicumarol bleeding?

Best No after irradiation

Cronkite I am sorry I dont get your point.

Barker You mean the specific bleeding into the sinuses of the lymph nodes?

Best Yes Is it more easily corrected by the platelets than bleeding elsewhere?

Cronkite I dont know how to answer that I can say that by putting platelets back in you dont get that type of bleeding

Best At the same time do you prevent bleeding elsewhere?

Cronkite Yes

Best Then there is no difference

Warner The very diffuseness of this bleeding into the sinusoids of the lymph node would suggest that it is perhaps not quite the usual hemorrhage which should be more or less accidental and spotty as it is in the dicumarol treated animal

Cronkite To diverge for a moment we have gone back into rather ancient morbid anatomical literature trying to find some good descriptions of human lymph nodes in thrombocytopenic purpura and aplastic anemia We cannot find anywhere in the older German literature a record that anybody has systematically dissected out the lymph nodes in bleeding diseases other than in the irradiated animal The latter has been done very well by many people who are all in agreement on the appearance Perhaps you or some of the other pathologists have studied thrombopenic purpura and dissected out lymph nodes from throughout the body and made a good histologic study of them

Warner I havent and I have not seen any reports on it

Flynn I have never done it

Tocantins Stewart and I did it in thrombocytopenic purpura in animals and found the same thing

Cronkite Yes I know I am wondering about idiopathic purpura and the primary refractory anemias and so on We have not yet found it in the literature

Tocantins The same sort of thing is seen in hemorrhage into the abdominal cavity not connected with thrombocytopenic purpura Often the lymph nodes of the mesentery show what may be called a streaked "mosaic" appearance They look striated from the outside And in histologic sections the cortical sinuses are full of blood this resulting simply from the drainage of blood extravasated into the peritoneal cavity

Cronkite I think there is truly a problem in just the histologic study of different types of hemorrhagic disorders in man and animals

Flynn Dr Allen raised the question of a shunt developing between the vascular bed and the lymph bed permitting blood to enter the lymph sinuses and thus accounting for the hemorrhagic appearance of the lymph nodes By a shunt most biologists refer to a blood vessel containing smooth muscle within its wall (i e an arteriole) which is interposed between the arterial and venous circulation permitting blood to bypass the capillary network between the two There is no question that shunts do exist in various parts of the body but I think it unlikely that they develop because of irradiation per se If in irradiation an abnormal drainage does occur it most likely results from abnormal permeability of existing channels (probably on the order of capillaries) rather than the development of true anatomical shunts

Cronkite I am in complete agreement with you Dr Flynn I think abnormal permeability is what happens however it has not been proved Dr Brecher has some histologic evidence for the presence of tiny vessels traversing the cortical sinus If the integrity of these vessels is lost there will be frank bleeding into the cortical sinus because the pressure in the blood vessels will be greater than in the lymph vessels I personally cannot visualize any better explanation but do not know why it should happen

Barker I dont see how you can get away from the possibility of drainage through the lymphatic vessels of very small amounts of blood which have been slowly extravasated into the tissue spaces distal to the nodes

Cronkite You get the previous picture in the lymph node prior to the occurrence of visible bleeding anywhere else in the body

Bright red lymph nodes are found that drain an area Search of the whole area fails to reveal obvious petechiae or hematoma

Alexander Are similar phenomena seen in the spleen?

Allen No

Alexander That is an extraordinary thing

Allen Well it is and it isn t

I have been wondering about this and I should like to ask some of you about it what is the possibility of the lymphatics draining the spleen? That is to what if any extent does the spleen have a separate lymphatic circulation?

Tocantins There are no lymphatics in the spleen

Allen That is what I wanted to hear somebody say And actually Dr Cronkite is it not correct that the spleen for the most part rather than being engorged is atrophied?

Cronkite The lymphatic portion is atrophied I don t want to talk for Dr Brecher who does the morphology with us but in a spleen I for one don t know enough about the actual relationship of the sinusoids to the circulation to tell whether there is or is not hemorrhage or whether blood is just pooled there I cannot tell histologically

Allen You would agree that it is an entirely different picture from this?

Cronkite Oh yes

At the present time it is not possible to come to any definite conclusions about why the irradiated animals bleed The bleeding is very closely correlated with the thrombocytopenia and can be prevented by platelet transfusions even when inadequate platelet levels are maintained and when the coagulation defect is not completely corrected to the normal for prothrombin utilization

Another consideration that should be mentioned is the epinephrine potentiating effect of sequestrene (40) the anticoagulant that is used for collecting platelets Rutin and flavonones possess this effect and have been described as preventing radiation hemorrhage (41) but this was not confirmed by Kohn *et al* (42) and Cronkite *et al* (43 44) Since the control animals received platelet free plasma in amounts equal to the plasma given with the platelets containing an identical amount of sequestrene it is thought that this factor is not responsible for the beneficial effect of the platelet transfusions

Tocantins Did the platelet poor plasma in the control animals contain sequestrene?

Cronkite Yes

In conclusion the induction of almost a complete absence of platelets as can be done by sufficient irradiation and time produces a very serious defect in the hemostatic mechanism by loss of the following functions ascribed to platelets

1) The ability to form platelet plugs
2) The vasoconstrictor substance of platelets [Zucker (45)]
3) The possible interaction of platelets with an antihemophilic factor or other plasma constituents to initiate coagulation
4) The acceleration of prothrombin conversion (37)
5) Platelet accelerator for first and second stage of coagulation and the platelet clottable factor (46)
6) Initiation of clot retraction

The complete removal of the above certainly seriously weakens the defenses against spontaneous bleeding makes wound hemostasis less effective and may be sufficient in itself to produce spontaneous purpura Whether there are other factors that contribute to the bleeding state in the irradiated animal must remain an open question for the time being There is still much work to be done

I should add that platelet transfusions separated platelets transfused into normal animals are not always innocuous Occasionally one sees a violent reaction of the anaphylactic type in which there is incontinence of urine and feces and there is bronchial spasm Whether this is due to the type of platelets or what the explanation is we do not know When precautions are taken to assure asepsis and pyrogen free equipment violent reactions may still occur I do not want anybody to get the idea that platelet transfusions are completely innocuous

Perhaps the most important phase of the irradiation work is the development of a test object namely the irradiated dog in which one can study platelet functions and clotting mechanisms in the virtual absence of platelets The intensive study of radiation has been forced by the development of atomic weapons and the uncertainty of diplomacy as a solution to international political disputes However the fortunate side of this practical problem has been the development of the irradiated dog that is not only excellent for the study of problems concerned with hemostasis but also for factors controlling the regeneration of hemopoietic tissues the gastrointestinal trace and the gonads Radiation has become a most valuable tool in the study of fundamental problems of biology and medicine This will be one of the everlasting contributions forced upon many laboratories by the hazards of possible atomic warfare

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THE PATHOGENESIS OF IRRADIATION HEMORRHAGE

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THE CLINICAL PATTERNS of postirradiation hemorrhage and pancytopenia are sufficiently constant in dogs exposed to total body x irradiation at a dosage of approximately twice the LD_{50} that the course of most animals so exposed can be predicted with reasonable certainty All develop severe thrombocytopenia leucopenia and anemia by the tenth to twelfth day after irradiation and die within thirty days The average duration of postirradiation survival at 450 r total body exposure under the conditions of our laboratory is eleven days Premortal hyperexia is an invariable finding in untreated animals Weight loss progresses without interruption until death in general the animals lose 15 to 17 per cent of their pre irradiation weight

The onset of hemorrhage may appear as early as the fifth post irradiation day but generally bleeding is not evident until the eighth or ninth day The extent of external hemorrhage (oral and gastrointestinal) varies to a considerable degree Although bleeding may occur into any organ it is more frequently and extensively observed about the teeth from ulceration of the buccal and gastro intestinal mucosa and in the lungs The incidence in which hemorrhage has been noted grossly in various organs after 450 r x irradiation is tabulated in Table XIV

The tendency toward pancytopenia for a group of animals is statistically evident within forty eight hours after x irradiation and can be demonstrated for the individual animal by the fourth to fifth day This hemocytologic picture is unfailing and at this level of exposure seems to depend only upon the animals surviving long enough for these changes to appear Of interest to us has been the observation that the development of anemia is a constant finding and that the extent of the anemia need not parallel the extent of external or grossly evident hemorrhage It should be pointed out however that most of the tissues and organs show some evidence of hemorrhage microscopically Hence the possibility that hemorrhage is still an important factor in causing the anemia cannot be

TABLE XIV

Location and Frequency of Gross Hemorrhage of Certain Areas in the 450 R X Irradiated Dogs

Location of Hemorrhage	Frequency of hemorrhage in untreated dogs (%)
Skin (other than venepuncture sites)	28
Gingiva	50
Chest	
Parietal Pleura	69
Mediastinum	43
Lungs	
Hilar Hemorrhage	49
Pneumonia	82
Heart	
Pericardium	53
Epicardium	66
Diaphragm	27
Abdomen	
Retroperitoneal	26
Stomach	52
Intestine	77
Colon	61
Kidneys	25
Bladder	32

excluded If hemorrhage is the principal cause of anemia it must be concluded that blood loss through microscopic hemorrhage is more important than that lost from grossly evident bleeding That a factor other than hemorrhage also contributes to anemia is clear from the following information Dogs from which 25 to 35 ml of blood have been drawn daily for ten days to two weeks after x irradiation develop no more extensive anemia than animals from whom only one or two samples of similar volume are removed over the same period of time

The damage produced by total body irradiation is so far reaching that most if not all of the clotting mechanism may be directly or indirectly involved In addition there is evidence of extensive vascular damage, about which little is known Since the normal hemostatic mechanism is not well understood and the effects of x irradiation are only partly known rational and complete treatment of irradiation hemorrhage does not yet exist However certain features of this syndrome are established and limited therapeutic aid is available

Irradiation hemorrhage was said to be the frequent cause of death in those who died from irradiation injury in Japan (1) As our information has been extended experimentally we have questioned the validity of this conclusion In dogs subjected to lethal (450 r) exposures of x ray given over the entire body hemorrhage is generally the most obvious gross finding but it is doubtful if hemorrhage often is an exclusive cause of death This complication is an important factor in morbidity if not in mortality but it may exert its deleterious influence by affording nidi from which fatal infection may commence more often than death is caused from any direct effect of blood loss

Among the disturbances in the hemostatic mechanism of the dog following 450 r total body exposure to x irradiation are thrombocytopenia an increase in the whole blood clotting time and the bleeding time a reduction in prothrombin consumption an increase in concentration of fibrinogen and a decrease in capillary fragility The activities of prothrombin of Ac globulin and spca do not appear greatly disturbed In addition there is evidence suggesting denaturation of plasma proteins which may or may not influence coagulation Except for thrombocytopenia the changes in most of the components of coagulation after irradiation exposure follow no absolute pattern In one animal a particular hemostatic disturbance may be more in evidence than in another Nearly all dogs however show an increased whole blood clotting time during the bleeding period

At 450 r exposures dogs invariably develop severe thrombocytopenia if they survive seven days or longer Thrombocytopenia is classically associated with petechial hemorrhages a prolonged bleeding time and impaired clot retraction These changes are attributed to platelet deficiency Although it is not understood how a deficiency in platelets accounts for the capillary fragility associated with thrombocytopenia it is to be expected that if thrombocytopenia is important in the pathogenesis of abnormal bleeding in Werlhof's disease in man it also should be important in the pathogenesis of irradiation hemorrhage To a certain extent this comparison may not be valid principally because in Werlhof's disease the marrow appears normal or hyperplastic whereas in irradiation thrombocytopenia the marrow is hypoplastic or aplastic What if any bearing this difference in marrow appearance may play in the etiology of these hemorrhagic disorders remains to be seen

Platelets are important to normal hemostasis because they form the initial thrombus at sites of vascular injury serve as a foundation

to anchor the fibrin clot and are a source of thromboplastin activity One of the reasons for the prolonged bleeding time in thrombocytopenia is the longer time required for a platelet thrombus to form when the circulating blood is thrombocytopenic Platelet deficiency however does not explain the increased fragility of the capillary walls unless it is assumed that in some unknown manner the platelets constantly contribute to the integrity of the vascular endothelium or cement substance Thus far there is no direct evidence in support of this possibility although indirect evidence is abundant

The lymph nodes draining various areas of the body become engorged with erythrocytes as they filter the bloody lymphatic circulation This phenomenon has been extensively studied by Ross Furth and Bigelow (2) In our experience the more extensive the petechial bleeding the more extensively engorged will be the lymph nodes draining these areas Because petechial bleeding is usually more pronounced in the gastrointestinal tract the mesenteric and periaortic nodes draining these areas are usually more prominently engorged with blood than are other nodes from other areas although the nodes from all areas are usually involved to some extent

The explanation for the engorgement of the lymphatic circulation with blood after irradiation is related to thrombocytopenia The transfusion of platelets in sufficient numbers to prevent serious thrombocytopenia prevents most of the petechial hemorrhages as well as the engorgement of the lymph nodes as Cronkite has shown here today and which we have confirmed Of interest is the fact that the spleen which has no independent lymphatic circulation is small and not engorged with blood after irradiation It appears to us that the blood filled lymphatic circulation is a manifestation of the ease with which the red cells may pass into the extravascular spaces and lymphatics Its correction or prevention by platelet transfusions illustrates particularly well an interrelationship between the platelet count and capillary fragility and strongly suggests that one of the normal functions of the platelets is concerned with maintenance of the integrity of the small vessel or capillary circulation It is further evidence that the functions of elements of coagulation are dynamic not static as generally considered (3) Relating the blood engorgement of the lymphatics to petechial hemorrhage of the areas drained and the petechial hemorrhages to the platelet count seriously questions whether the capillary damage in total body irradiation within the $LD_{50\ 100}$ range is the result of a direct insult to these structures as implied in the past More likely the capillary

fragility is secondary to suppression of the platelets

At first inspection these data suggest platelet transfusion as an antihemorrhagic measure This hope is soon despaired of however when one realizes the enormous number of platelets necessary to replenish the platelet number in the irradiated thrombocytopenic state and that within a matter of hours they all will have disappeared

Following 450 r x irradiation dogs frequently develop ulcerations throughout the alimentary tract These lesions appear between the first and third week when the animal is thrombocytopenic and very susceptible to infection Undoubtedly any abnormality in the clotting mechanism would increase the extent of bleeding from such lesions thrombocytopenia being no exception

It is doubtful that the ulcerations of the gastrointestinal tract arise primarily from thrombocytopenia Animals receiving less irradiation (175 to 275 r) and surviving still develop thrombocytopenia comparable to that seen at exposures of 450 r but they usually do not develop alimentary ulcerations and have no external evidence of spontaneous bleeding Animals exposed to 450 r whose heads have been shielded by lead develop severe thrombocytopenia but nearly three fourths of these have shown little evidence of alimentary hemorrhage when sacrificed between the seventh and fifteenth day after exposure If the remainder of the animal was shielded 450 r x radiation of the gastrointestinal tract failed to produce alimentary ulcerations

The tissues responsible for the prevention of these ulcerations appear to be the marrow and the reticuloendothelial system When the skull liver or pelvis of the dog is shielded by three sixteenths of an inch of lead the thrombocytopenia and leukopenia are just as severe as in the unprotected animal but the rate of return to normal of both the platelet and leucocyte counts is much more rapid (Figures 93 and 94) Recovery begins at the end of two weeks and is nearly complete at the end of three weeks in the shielded animal in contrast to five to seven weeks in the unprotected animals The great reduction in the number of alimentary ulcerations and the reduction of gastrointestinal hemorrhage in the head shielded thrombocytopenic animals suggest an intimate though undetermined relationship between marrow function and the ulcerations of the gastrointestinal tract in irradiation injury which should be investigated further

In the last analysis the most important single defect in the clotting mechanism contributing to irradiation hemorrhage is

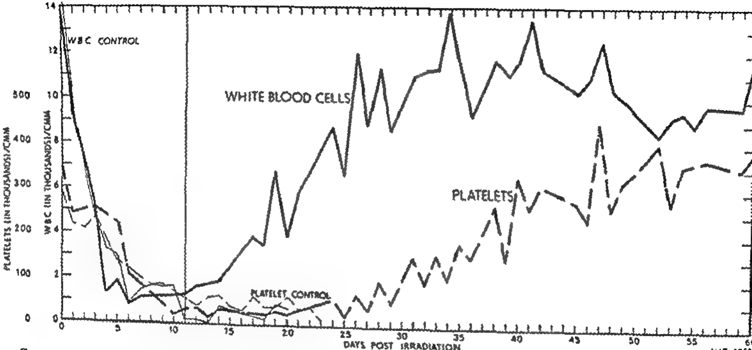


Figure 93 Accelerated rate of recovery of platelet and white blood counts following total body irradiation with head shielded Compare with Figure 92

DOG 462 IRRADIATED 450R X RAY

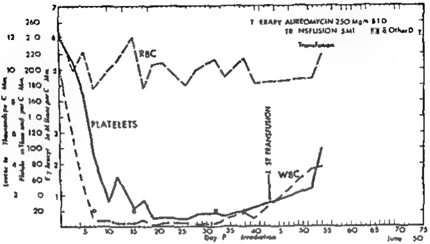


FIGURE 94 Delay in platelet recovery for five to six weeks after 450 r exposure in a dog which survived

probably thrombocytopenia If thrombocytopenia could be prevented it is probable that the complication of irradiation hemorrhage would disappear Much of the confusion existing today in the field of irradiation hemorrhage can be attributed to inadequate knowledge and appreciation of platelet function It is well to list the functions as known thus far again (*a*) platelet agglutination and adherence to the site of vascular injury (*b*) as a source of thromboplastin (*c*) as an accelerator of prothrombin conversion (*d*) as an effective antiheparin (*e*) as contributing in some unknown way to the integrity of the vessel wall and (*f*) possibly several other unknown functions Hence when thrombocytopenia is present nearly all phases of coagulation are disturbed Since these different clotting disturbances vary in extent from one animal to another and are magnified by thrombocytopenia the pattern of the hemorrhagic syndrome and of the changes in the clotting mechanism can be expected to be inconsistent and difficult to analyze The problem is further complicated because some of the changes observed in the clotting system after irradiation can be masked by elevating the platelet count with platelet transfusions From a practical standpoint however the correction of platelet deficiency will almost certainly reduce to a minimum if not abolish the hemorrhagic syndrome associated with irradiation

WHOLE BLOOD CLOTTING TIME

In some animals the clotting time is prolonged more than in others The lengthened clotting time usually does not make its appearance before the second week following 450 r exposures in dogs The blood is rarely incoagulable unless the animal has received frequent blood transfusions Usually the clotting time is prolonged two or three times the normal control value Once the clotting time is increased it does not necessarily remain abnormal until death Normal values are sporadically observed in some animals particularly during the preterminal state In Figure 95 are

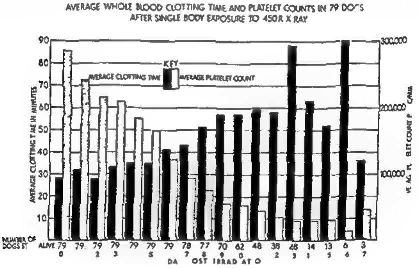


FIGURE 95

presented the average clotting time values of seventy nine consecutively irradiated dogs in which no form of therapy was used On only three occasions in the group of 665 clotting time determinations carried out on these animals was the blood found to be incoagulable Five of these seventy nine animals revealed no detectable increase in the clotting time at any point of study during the irradiation period

Some workers have failed to detect any increase in clotting time in dogs following irradiation (4 5) These differences in observations can be attributed to the fact that such reports are based on the examination of only a few animals also the increase in clotting time of the magnitude observed in this laboratory can be easily obscured by the troublesome clot accelerations incident to the

usual clotting time techniques No method yet devised as a measure of the *in vitro* clotting time of blood can be construed to reflect accurately the status of *in vivo* coagulation or to be free of accelerating artifacts The clotting time is of value only when these artifacts are reduced to a minimum and under these conditions some abnormalities can be detected following total body x irradiation

A statistical analysis of the 79 irradiated dogs alluded to above has been carried out by Dr L J Savage of the Department of Mathematics of the University of Chicago These animals were irradiated and studied between July 1 1948 and February 1 1950 This period was chosen because it included a reasonably large homogenous body of data under nearly constant laboratory conditions For this group the mean clotting time was 28 5 minutes which with ninety nine per cent certainty may be said to lie within 1 8 minutes of the stated figure

Information on the variabilities of repeated control determinations of the same dog may be summarized as follows

	Estimate	99% Confidence Interval
Variance	31 50 min	28 54 to 34 53
Standard Deviation	5 61 min	5 34 to 5 88

The variability of the clotting time attributable to differences between dogs during the control period was actually less than the variability of multiple determinations on the same animal

	Estimate	99% Confidence Interval
Variance	4 67 min	0 00 to 6 62
Standard Deviation	2 16 min	0 00 to 2 57

Returning to the estimate of 5 61 minutes for the standard deviation of measurements on one dog under control conditions it can be roughly concluded that if N dogs (N 5) are measured once each in the control and in some treated state and if the treatment induces a change d in the mean clotting time that there are about nine chances in ten of detecting the change (and calling it significant at the 05 level) provided d is even as great as d $= 3\,28 \times \sqrt{\frac{2}{N}} \times 5\,61$ i e d $= 25\,95 / \sqrt{N}$ min For example

N	9	16	25	49	100
d	8 7	6 5	5 2	3 7	2 6 min

Estimates of mean and standard deviation from dog to dog were computed for several postirradiation days and are presented in Table XV Under the conditions of the experiment the standard

TABLE XV

Mean Clotting Times and Standard Deviations in Postirradiation Clotting Times of Dogs

Day	1	2	3	4	5	6	7	8	9	10	11
Number of dogs observed	43	30	39	32	40	38	41	38	24	28	20
Mean clotting time	30.65	30.53	31.74	35.12	34.50	42.08	46.71	50.34	50.29	43.57	54.90
Standard Deviation	7.12	9.25	6.35	8.44	7.82	19.3	18.00	23.98	21.10	17.75	22.60

deviation tends to increase very rapidly with the mean. The relationship between the mean and standard deviation is roughly given by the following equation, estimated graphically from Table XVI:

$$\begin{aligned} S.D. &= 0.87\,m - 1.95 \\ &= 0.87\,(m - 2.23) \end{aligned}$$

TABLE XVI

Three-day Comparison of Clotting Times with Control Periods

Day	Higher	Lower	P
1	40	20	.02
8	58	9	10^{-8}
12	30	5	10^{-4}

Table XV and, equivalently, the equation above, show that considerably larger variations in the clotting time may be expected when the mean value is considerably elevated. This has the practical consequence that a given absolute, and to a lesser extent relative, difference between two similar treatments will become harder to detect as the mean clotting time induced by the treatments increases.

This larger variation when the mean value is elevated is in a large part the result of minor changes in thromboplastic substances produced by imperfect venepuncture or handling of the blood, as illustrated by Figure 96. The factors which exert an accelerating effect on the clotting time have a greater influence when the clotting time is prolonged than when it is normal.

Figure 95 makes clear that clotting times tend to be elevated after irradiation, except possibly during the first few days and also during the preterminal state when too few dogs remain alive to provide adequate data. Objective support of this contention is provided in Table XVI, which shows, for each of three days, how many dogs had longer and how many shorter clotting times than in their control periods. These numbers depart from the 50:50 ratio which would be expected if irradiation were without effect on the clotting time. The probabilities of departure as great as that observed occurring on the basis of chance alone are also shown.

There is some evidence from Table XVI that the clotting time is increased even on the first day after exposure. By the eighth day

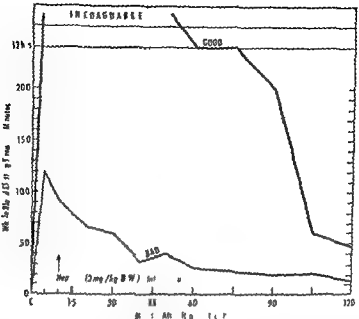


FIGURE 96 Clotting time differences obtained on the same dog after heparin administration by an investigator experienced in good venepuncture technique and by one who is not

the validity of the clotting time increase is indisputable and on the twelfth day is still excellent Though further evidence is not called for it may be of interest to note that while no clotting time in the control period was as high as 45 minutes 74 of the 79 dogs attained or exceeded this value some time after irradiation

From these data it is beyond doubt that the evidence pointing to increased clotting time after irradiation is not attributable to chance alone A number of possibilities arise which may explain the increased whole blood clotting time following irradiation Most prominent among these are (*a*) thrombocytopenia (*b*) a delay in the rate of conversion of prothrombin to thrombin (*c*) a circulating anticoagulant and (*d*) denaturation of the plasma proteins concerned with coagulation

THROMBOCYTOPENIA AND WHOLE BLOOD CLOTTING TIME

It is generally held that thrombocytopenia increases the bleeding time but does not alter the clotting time Because of the difficulties encountered in explaining the cause of the increased whole blood clotting time after irradiation the relationship of thrombocytopenia to the clotting time has been studied In Figure 95 it will be noted that the decline in platelet count is essentially the reciprocal of the

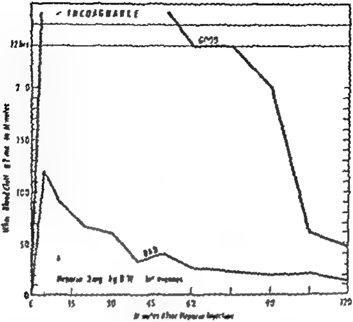
increase in clotting time The data presented in this chart are mean values of clotting times and platelet counts and for this reason obscure the fact that increases in the clotting time in the individual animal do not necessarily parallel the development of thrombocytopenia i e the platelet count and whole blood clotting times do not always parallel one another These observations are in accord with those on human patients where it is known that thrombocytopenia can exist without evidence of spontaneous bleeding Furthermore when the clotting time is prolonged and capable of responding to the administration of toluidine blue it does so without altering the platelet count and bleeding under these conditions may be temporarily controlled even though thrombocytopenia persists

That thrombocytopenia and the increased whole blood clotting time are independent phenomena is apparent from irradiation studies in which the animal survives At 450 r exposure some dogs will survive when given aureomycin in combination with fresh blood transfusions (6) In these animals the platelet count returns slowly to normal (Figure 94) In general there is no appreciable recovery of the platelet count short of four to five weeks On the other hand, the whole blood clotting time usually returns to normal by the eighteenth day approximately ten days before any recovery of the platelet count can be detected When the head of the dog is shielded with lead and the remainder of the animal exposed to 450 r the course of events is essentially the same with the exception that the platelet count begins to recover after two weeks and reaches normal by the third week. Curiously the shielded animals which do not survive die with the same hemorrhagic manifestations seen in the unprotected irradiated dogs Severe thrombocytopenia is the constant finding in all dogs receiving 450 r total body x irradiation in contrast to the less constant increases in the whole blood clotting time which may vary considerably from one animal to another and indeed from day to day in the same animal

It is possible that there are qualitative as well as quantitative platelet changes after irradiation and that alterations in kind rather than in platelet number may relate to the variations in clotting time This aspect of the platelet problem has not been studied

RATE OF PROTHROMBIN CONVERSION AND WHOLE BLOOD CLOTTING TIME

The rate of conversion of prothrombin to thrombin is reduced in most animals after irradiation (7) This may be detected by stopping the clotting process at the end of one hour by addition



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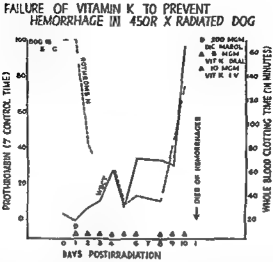

FIGURE 97 Experiment showing the ability to form prothrombin during the period of irradiation hemorrhage Note that the prothrombin activity returned to normal during the same period the whole blood clotting time was lengthening and a thrombocytopenia was developing

the animal was irradiated This dose of dicumarol produced profound reduction in prothrombin activity which returned to normal during the period in which the whole blood clotting time was lengthening and as thrombocytopenia developed

A clotting inhibitor can be demonstrated in the plasma of a small percentage of dogs receiving a total body exposure of 450 r x ray When present it is generally weak and difficult to ascertain In the course of study of this problem in our laboratory it became evident that in attempting to completely control our earlier work by replacing blood as each day s sample was withdrawn we may have introduced another problem that of transfusion reaction In some animals transfusion reactions occur even when the blood has been properly cross matched The reactions noted have been anaphylactoid in nature Since the immune mechanism is disturbed after irradiation it is also possible that certain incompatibilities may arise from blood and that these may not be recognized by the usual techniques In fact denaturation of plasma proteins after irradiation is a possible source of these anaphylactic reactions and they may lie within the plasma and be unrelated to any incompatibility of the red cells It is necessary therefore to study the clotting mechanism in those animals which have been transfused and in those which have received no blood

of sodium oxalate The remaining plasma is then assayed for its prothrombin activity In some animals nearly ninety per cent of the prothrombin activity remains In the normal animals nearly ninety per cent of the prothrombin activity has been consumed in this same period of time

Further evidence suggesting a diminution in the rate of thrombin formation can be detected by periodically removing the fibrin clot as it forms Fibrin begins to make its appearance within thirty to fifty minutes after the blood sample is withdrawn A small wooden applicator introduced into the tube containing the blood and rotated to remove fibrin as it is formed reveals that not all of the fibrin may be removed over a period of one to three hours after irradiation whereas complete defibrination of normal blood by this process may occur in less than one hour

The apparent rate of thrombin and fibrin formation is not the result of prothrombin deficiency or of a diminished concentration of plasma fibrinogen After irradiation there is no important or constant alteration in the activity of plasma prothrombin The concentration of fibrinogen is generally increased above that of the pre irradiated control period Two obvious possibilities arise which could explain the delay in prothrombin conversion and fibrin formation Since all the animals are thrombocytopenic they are deficient in at least one source of their thromboplastin activity This reduces the rate at which thrombin is formed and in turn the rate at which fibrin appears It is also possible that they are deficient in accelerator globulin a deficiency which likewise retards thrombin formation A study of Ac globulin activity however fails to reveal any reduction in this factor Further support of a normal activity of Ac globulin is the observation that fresh whole blood transfusions containing this factor exert no consistent effect on the prothrombin time the whole blood clotting time or the bleeding syndrome in the irradiated animal

PROTHROMBIN ACTIVITY AND WHOLE BLOOD CLOTTING TIME

By the one stage prothrombin procedure the prothrombin activity has remained normal, or nearly so throughout the course of the irradiated animals Moreover the irradiated animal appears able to form prothrombin when the whole blood clotting time is greatly prolonged and during the period of irradiation hemorrhage Figure 97 shows the suppression of prothrombin activity following the administration of a single dose of dicumarol given the same day

and that of the serial dilution of the irradiated animal s blood with normal saline Moderate dilution shortens the clotting time when an anticoagulant is present but as further dilution is carried out the clotting time is prolonged Dilution of normal blood in this manner does not shorten the clotting time until the blood has been diluted three to five times the normal value The difference in the two clotting time responses to normal blood and to blood containing an anticoagulant seems to be explained on the basis that the effect of the anticoagulant is diluted out before the normal excesses of the clotting mechanism are appreciably affected

Recently we have made a study of the efficiency in our hands of the Charles and Scott heparin extraction procedure for normal blood to which heparin has been added The control extractions included blood to which 20 mg of Abbott s liquid commercial heparin had been added to each 100 ml of blood 10 mg to each 100 ml of blood and 1 mg of heparin per 100 ml of blood The percentage of the added heparin recovered was 11 2 when 20 mg per cent had been added Of heparin 0 7 was obtained when 10 mg per cent had been added No heparin was recovered when 1 mg per cent had been added Using this same lot of heparin 2 to 4 micrograms were sufficient to increase the clotting time of 1 ml of normal dog blood 5 to 6 micrograms per ml rendered the blood incoagulable It is clearly evident that the sensitivity of the extraction procedure in our hands was insufficient to obtain any heparin had it been present in the bloods in which coagulation was delayed but not present in our irradiated dogs If heparin is the anticoagulant detected in our assay procedure and if it is of a potency comparable to that of Abbott s liquid commercial heparin (100 to 130 units per ml) the amount of this anticoagulant necessary to increase the clotting times to the extent generally observed in our irradiated dogs was exceedingly small It will be recalled that when these animals became thrombocytopenic their sensitivity to added heparin was reduced to about one twentieth or one thirtieth that tolerated by normal blood Assuming heparin to be the anticoagulant and to be of high potency as little as 0 00025 to 0 00030 mg per ml would be sufficient to cause the clotting time changes noted Since the method in our hands yielded no extractable heparin when less than 10 00 mg of heparin per 100 ml of blood was present it is obvious that the extraction procedure could not have detected heparin in the blood had it been present in our irradiated animal

A review of our data in which a clotting inhibitor responding to toluidine blue or protamine sulfate could be demonstrated reveals

Our initial experiments begun in 1945 were designed to determine whether the hemorrhagic syndrome following total body x irradiation could be prevented by the daily transfusion of whole blood In this respect transfusion proved useless Because the irradiated 450 r dog develops a profound anemia and because our studies required the withdrawal of 25 to 30 ml of blood daily it seemed desirable that the blood be replaced after each sample was removed lest the animal die of anemia of irradiation compounded by that of blood loss incident to our studies

Dog 108 received blood replacement daily and represents perhaps the most striking example that we have yet encountered in which an anticoagulant could be demonstrated These dramatic findings do not represent the usual course following transfusion Caution is necessary in order that the data from this one animal are not interpreted as characteristic of all irradiated transfused animals The whole blood clotting time in dog 108 first became prolonged on the sixth day and by the ninth day the blood was incoagulable At this time it could not be made to clot even though beef lung thromboplastin was added Four samples of blood drawn on the ninth day all showed this same phenomenon Thus the validity of the observation was established and it cannot be construed as artifact During the stage of incoagulability blood and plasma from this animal were mixed in varying concentrations with blood and plasma of a normal dog a procedure which delayed the clotting of both normal plasma and normal blood Since heparin is the best known endogenous anticoagulant this possibility was examined Toluidine blue and protamine sulfate which are antiheparins were added to different samples of incoagulable blood After incubation at 37 C the blood clotted within ten minutes The animal was then given 75 mg of toluidine blue intravenously and the extensive bleeding about the mouth and from the previous puncture wounds ceased within less than an hour The clotting time was returned to normal values and remained normal on the five occasions it was examined prior to the animal's death fifteen hours later

In the blood of animals studied subsequently an incoagulable state was still observed but not as frequently as anticipated The whole blood clotting time was found to be increased in most animals when a careful technique was employed in the conduct of this determination Of ten animals consecutively studied only three showed evidence of anticoagulant activity in blood (8) Two types of tests for anticoagulant activity in blood were employed that of incubating the blood of the irradiated animal with normal blood

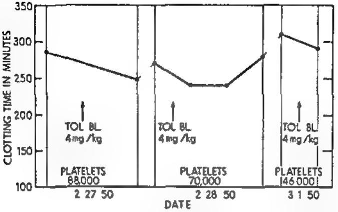

FIGURE 99 The arrows indicate the administration of toluidine blue

July of 1947 we have not observed a clotting inhibitor that responded to toluidine blue or protamine sulfate except when transfusions had been given Although a clotting inhibitor can be demonstrated in the blood of the untransfused irradiated dog this one is quite different from that observed in those animals which were also transfused

Only a small percentage of the irradiated animals transfused displayed evidence of an anticoagulant responding to the administration of toluidine blue or protamine sulfate Most but not all of these animals displayed some evidence of reaction to the blood administered In some an accelerated rate of clot lysis also occurred and this was associated with a transient reduction in fibrinogen concentration

Thus in our animals there have been observed two types of anticoagulants One appears with some degree of regularity and does not respond to toluidine blue or protamine sulfate The other (previously described) is related to the frequent transfusion of blood appears sporadically and does respond to toluidine blue and protamine sulfate The clotting disturbance in this latter group resembles but is by no means identical with that produced by heparin

PROTAMINE TITRATION

The difficulties encountered in extracting blood for small amounts of heparin led us to employ a bioassay procedure for heparin based on the quantitative relationships between heparin concentra

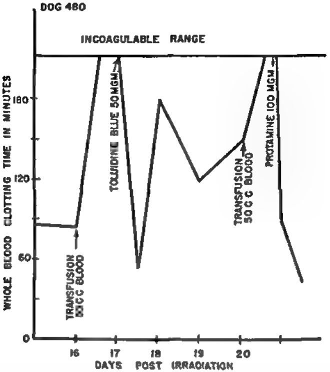

FIGURE 98 Clotting time in this irradiated animal was increased after blood transfusion and returned to pretransfusion level when toluidine blue and protamine sulfate were given Reprinted by permission from Allen J G Moulder P V and Enerson D M Pathogenesis and treatment of the postirradiation syndrome *J A M A* 145 *701 (1951)*

that this was a phenomenon observed only when the irradiated animal had been frequently transfused Illustrating this point are the data presented in Figures 98 and 99 The clotting times were prolonged in both animals but in the case of the data presented in Figure 98 the blood incoagulable following transfusion promptly responded to the administration of toluidine blue and protamine sulfate In the animal whose data are presented in Figure 99 and which received no transfusion the administration of toluidine blue on three different occasions did not influence the whole blood clotting time

Until July of 1947 all of our animals received transfusions each day blood was withdrawn and in quantities equal to the blood withdrawn Because this was a troublesome procedure and influenced neither the extent of postirradiation anemia nor the rate of survival the procedure was abandoned except in those animals whose anemia became sufficiently severe to threaten anoxia Since

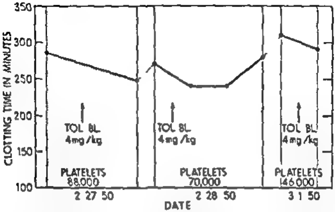

FIGURE 90. The arrows indicate the administration of toluidine blue

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TABLE XVII

In vitro Heparin Sensitivity of Whole Blood of Normal Dogs

		Micrograms of heparin per ml blood									
Dog No	Control	1	2	3	4	5	6	7	8	9	Control
571	8	—	14	43	67	80	6 hrs	6 hrs	—	—	9
575	15	—	48	74	6 hrs	6 hrs	6 hrs	—	—	—	15
[illegible]	15	25	37	63	84	94	113	218	15 hrs	—	23
625	15	44	49	80	143	246	6 hrs	6 hrs	6 hrs	—	16
460	15	15	51	135	135	375	375	375	375	18 hrs	15
699	18	16	39	39	49	49	105	140	445	445	12
717	12	22	26	52	200	420	420	24 hrs	24 hrs	24 hrs	17

tion and the clotting time of whole blood When heparin is added to blood the clotting time response is first small and then increases rapidly as shown in Table XVII Since 2 to 4 micrograms of heparin may be added to a milliliter of blood before the clotting time of normal blood is affected it seemed possible that the presence of endogenous heparin might be detected if its effect were summated with that of added heparin presuming the chemical structure and biologic activity of native endogenous heparin and that of commercial bovine heparin to be similar In consequence a standard amount of commercial heparin was added to a standard volume of blood and after mixing this blood was placed in each of ten serology tubes containing increasing increments of the heparin antagonist protamine sulfate This heparinized blood and protamine mixture was allowed to stand for one hour at which time coagulation occurred in those tubes containing enough protamine to neutralize the effect of the added heparin In practice 100 micrograms of heparin (Abbott's) per 11 ml of blood was mixed by inverting the tube fifteen times One milliliter of this mixture was placed in each of ten tubes containing protamine beginning with 20 micrograms and increasing in 20 microgram increments to a total of 200 micrograms in the tube containing the greatest amount of protamine For normal dog blood tubes containing 100 micrograms of protamine or less failed to clot at the end of an hour whereas those containing 120 micrograms or more coagulated These results obtained for normal animals were remarkably constant for any given lot of heparin or protamine sulfate The normal pattern however may vary if the potencies of either heparin or protamine change Hence each new batch of materials must be standardized to establish the end point

The results obtained from blood of irradiated dogs have been surprising and have been difficult to interpret In some irradiated animals coagulation did not occur excepting in the tubes containing 140 micrograms or more of protamine sulfate In a few animals the blood failed to clot even when 200 micrograms of protamine were added If the cause of this increased protamine requirement were due to the presence in blood of endogenous heparin of a potency similar to commercial heparin the bloods of the animals in any instance in which the protamine titration was increased 20 micrograms or more should have been incoagulable In contrast to this observation the increases in the protamine titration in some of the irradiated animals were accompanied by only a slight increase in the whole blood clotting time In a few instances the

clotting time has been normal when the protamine titration has been increased Several possible explanations for this discrepancy can be envisioned These include (*a*) that the blood contains a heparin like substance capable of binding protamine but that this substance has little if any anticoagulant potency, (*b*) that the material responsible is actually endogenous heparin but that its anticoagulant power is reduced because of a deficiency in the plasma cofactor essential for *heparin s anticoagulant action* (*c*) that the increased protamine binding power is due to the liberation of non heparinoid substances with little or no anticoagulant effect but which have the same capacity as heparin in binding protamine e g nucleic acid substances (*d*) that there was no increase in endogenous heparin *heparinoid, or nonheparinoid substances* but that the heparin protaminate complex was dissociated to an unusual degree after irradiation and under certain other conditions

These possibilities have been examined and the results reported elsewhere (9) A number of factors were found to influence the protamine titration and among these was irradiation injury In some irradiated animals an increase in protamine titration was corrected by the injection of either protamine sulfate or toluidine blue *It is our belief that the data provided by the protamine titration* while reproducible are not sufficiently well understood to permit an intelligent interpretation at this time

The results obtained in our studies and those of others indicate that the components of coagulation are affected in no standard pattern *after x irradiation In one animal a particular hemostatic* defect may be more in evidence than in another The only constant finding is the extensive thrombocytopenia Clinical evidence of bleeding on the other hand varies widely and in some animals gross evidence of bleeding is not present What then is the role *of thrombocytopenia in the pathogenesis of irradiation* hemorrhage? Thus question cannot be fully answered although there are many obvious means whereby platelet deficiency can account directly for spontaneous *bleeding or contribute to the continuation of hemorrhage* once bleeding has commenced Where lesions exist bleeding may occur because the structure of the platelet thrombus is too small and friable to control bleeding or because that thrombus which does form is not well anchored by platelet adherence to the vascular wall So far as is known these functions of platelets are not taken over by any other hemostatic component when thrombocytopenia develops The superficial ulcerations following irradiation which developed in the oral and alimentary tract of most irradiated

dogs are frequent sites of extensive bleeding In the author s opinion this type of bleeding is greatly influenced by the platelet count In the presence of serious reduction of platelet number there are not enough platelets available to form thrombi rapidly enough to prevent extensive bleeding from such ulcerative lesions and those that do form are easily dislodged or fragmented

It seems doubtful that thrombocytopenia is the sole factor responsible for the prolongation of the clotting time of whole blood in irradiation hemorrhage for reasons already indicated However when plasma has been rendered platelet poor by high speed centrifugation the rate of fibrin formation is greatly retarded Unfortunately it has not yet been possible to devise a consistent *in vitro* method by which whole blood can be rendered thrombocytopenic by centrifugation without the aid of anticoagulants to prevent clotting during these procedures Thus it has not been possible to study the effect of thrombocytopenia produced *in vitro* in otherwise normal blood

DENATURATION (?) OF PLASMA PROTEINS

It is well known that the length of the clotting time of blood (*in vitro*) depends in part on the wetting characteristics of the surface it contacts In general the less the degree of wetting the longer the clotting time will be Containers coated with paraffin silicon and other hydrophobic materials are not wet by blood allowing the platelets to remain within the boundary of the blood itself As a result the platelets rupture more slowly so that little or no thromboplastic precursors are liberated or activated It is for this reason that the clotting is greatly delayed under these circumstances providing of course that the blood sample is obtained with a minimum of trauma to the vessel and surrounding tissue

The possibility arises that surface tension of blood may be altered after irradiation and thereby gives rise to some of the clotting changes noted Detailed below are a series of experiments devised for the study of surface tension in the blood and plasma of dogs before and after irradiation

Surface tension (expressed as dynes per cm) was determined as the force required to pull a platinum ring of known circumference through the surface of heparinized plasma (0 091 mg bovine heparin per ml of blood) This was measured on a Christian Becker chainomatic surface tension balance which employs the principle of the DuNuoy ring

In a series of surface tension measurements on heparinized

plasma in normal dogs it was found that 53 to 55 dynes per cm was the force required to pull the platinum ring through the plasma surface under standard conditions (temperature and barometric pressure change being taken into account) The figures for normal human plasma were similar No appreciable deviations from this value were noted for bloods with prothrombin deficiency hemophilia or normal blood to which heparin had been added

The surface tension of the plasma of dogs exposed to 450 r total body x ray however did increase and readings of 56 to 60 dynes per cm were frequently reached during the latter half of the usual 10 to 14 days postexposure survival period For the most part this increase in surface tension paralleled the increase in the whole blood clotting time as exemplified in Figure 100 It will be noted

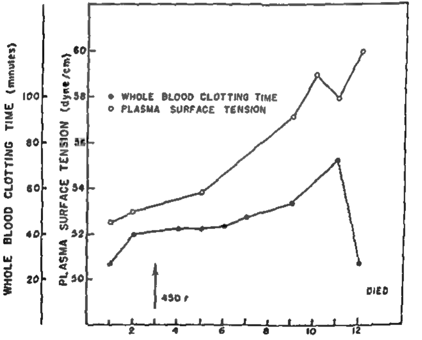


FIGURE 100

that in the terminal stages the whole blood clotting time occasionally dropped while the surface tension remained elevated Thus while the increased surface tension paralleled the clotting time in some instances there were obvious exceptions which suggested that this relationship was more apparent than real that it was subject

to limitations that seemed to render any correlation relatively meaningless.

It was considered that one of these limitations might be irradiation thrombocytopenia. However, changes in surface tension usually precede the development of thrombocytopenia by three or four days. Moreover, surface tension as measured on the Christian Becker instrument was not appreciably affected when normal heparinized plasma was depleted of platelets by high speed centrifugation.

It is possible that the increased surface tension of plasma accelerates the rate of platelet lysis and thereby accounts for the preterminal shortening of the whole blood clotting time.

A study was undertaken to determine whether any particular component of plasma was changed sufficiently to account for the alterations noted in surface tension. Proteins form the bulk of the colloids of plasma and are surface active. In addition, the lipid content of plasma, especially cholesterol, can alter surface tension activity. When the concentration of plasma protein is high, surface tension is low, and because of its smaller molecular size, albumin is more active than globulin. After irradiation, the concentration of albumin is depressed, but not to a sufficient degree to account for the changes noted. In some animals, surface tension was increased even though no reduction in plasma albumin occurred. Surface tension values comparable to those of normal plasma were obtained by adding 4 Gm. per cent of bovine albumin to normal saline. At 2 Gm. *per cent concentration the surface tension was* increased 1½ dynes per cm. The changes in surface tension after irradiation of the dog ranged between 2 and [illegible] dynes per cm. and do not seem explainable on the basis of changes noted in albumin or total protein concentration.

It appears likely that the increase in plasma surface tension after irradiation is due to alteration in the kind of protein rather than in concentration. Supporting this possibility is the increase in S-H groups following irradiation (9). It may be reasonable to assume that irradiation destroys, in part, the colloidal system of plasma, causing aggregation of the molecular dispersion system and thus reducing the number of surface activity particles.

To increase surface tension the plasma lipids should be reduced. However, following irradiation the plasma lipids are increased, suggesting that the lipid factors are of no great importance in this respect.

What, if any, connection can these physical changes in the protein moiety have upon the regulation of the clotting mechanism?

At this stage of development of our knowledge it can be argued that the changes noted in irradiation hemorrhage are so drastic that they may have no counterpart in clinical pathology It should be pointed out however that coagulation in the broad sense of the term represents the destruction of the colloidal system and this can be accomplished by a number of physical means as well as by certain biochemical enzymatic and immunologic reactions Denaturation of the plasma proteins could act to influence the clotting mechanism at any point where proteins are involved However the demonstration of an altered surface tension of plasma following irradiation does not establish by what means or to what extent changes in surface tension may contribute to the picture of irradiation hemorrhage The answer to this problem remains to be seen

Tocantins Have you been able to titrate heparin in the blood of the animals after they received blood transfusions?

Allen Yes we have biologically We have been able to get it very well We get an anticoagulant as potent as heparin

Brinkhous You are still referring to irradiated dogs?

Allen Yes However there is a hooker here because the irradiated animals are so to speak immunologically dead at the time when the anticoagulant is no longer apparent Actually when we go back over our data we find that the anticoagulant response begins not on the twelfth and thirteenth days but on the seventh eighth and ninth Therefore this reaction comes in the earlier phase in which the hematological situation is not yet completely altered

Jaques What do you mean by immunologically dead ?

Flynn Give an operational definition

Allen A rabbit is given an antigen his antibodies built up and then he is irradiated Ten days later when given the antigen he does not get much of a kick out of it although he will get a little But his unirradiated mate given the antigen may die So that the immunological response is greatly reduced at that point

Tocantins Dr Cronkite has sometimes observed an anaphylactic like reaction to the injection of platelets in some of his irradiated animals

Allen Those are agglutinated platelets wouldn t you say Dr Cronkite?

Cronkite In this respect I want to mention just one thing I had intended to speak about it yesterday during the discussion on dicumarol and the failure of transfusions occasionally to be of

benefit I say this with some hesitation because we have not dupli cated it enough to be certain of it The fresh platelets circulate in the normal animal This we know Every time platelets are trans fused a good rise in the platelet count is obtained But if those platelets sit around for twenty four hours in plasma at 5 C instead of getting a rise in the platelet count there is a thrombocytopenia and sometimes a vicious reaction I am hesitant to mention it but this may be an explanation for why bank blood sometimes produces more bleeding The changes that take place in plafelets while stored at 5 C for twenty four hours are not known Perhaps they become thrombocytolytic

Wright Are we to conclude Dr Allen that transfusion is actually harmful rather than beneficial for the treatment of irradiation injury?

Allen Yes in some animals at least Figure 101 is a bar graph

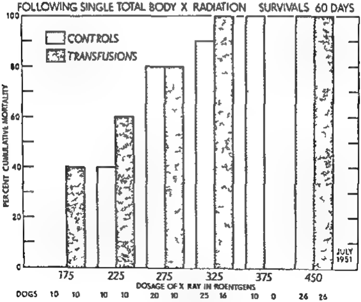


FIGURE 101

on the effect of transfusion of blood The figures speak for them selves It can be seen that in no column is there evidence of benefit from blood That is blood *alone* This point must be re emphasized because with blood and aureomycin after 450 r we have three survivals out of twenty

Cumulative Mortality of 450R X Ray Dogs

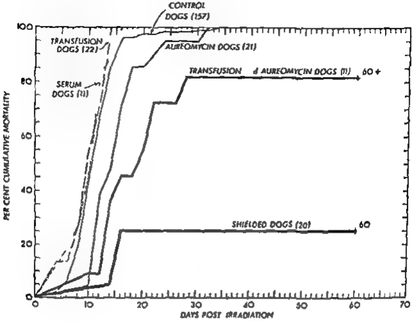


FIGURE 102

Alexander Is this fresh blood?

Allen This is fresh blood

Cronkite I object to your statement that transfusions are harmful to the irradiated dog Your data show that regular transfusions to maintain hemoglobin levels do not increase the survival rate In our experience in the irradiated dog blood transfusions are beneficial if given only when the animal is profoundly anemic Under these circumstances blood is a life saving measure

Allen Blood alone in our own experience and as far as I know in Joe Howland s * produces no benefit and he gets the same type of pattern that we do

Cronkite I am in complete agreement with your last statement I wonder if most people will recognize the terrific volume of work involved in what you have done We thought of it and we were afraid to undertake it It was just too much work

Allen It has taken two years

Wright What about aureomycin without the blood?

Allen There is some benefit but less than when aureomycin is combined with blood transfusion

* Personal communication

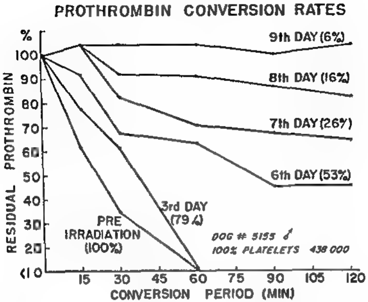


FIGURE 103 Normal dog 10 9 kg body weight given 300 r to each side with G E 2000 kv x ray unit Respective days of testing after irradiation indicated on each curve parenthetical figures indicate platelet concentration as percentage of pre irradiation level Dog died on tenth day

Cronkite We have not had a transfusion reaction when we wait until they get really anemic and desperately need blood Then we give them one big transfusion

Allen We don t either under those circumstances

Cronkite We have never given repeated blood transfusions With repeated platelet transfusions reactions are rare in the irradiated animal

Ferguson I suggest that we stay off the subject of transfusion reactions I would rather hear about radiation

Allen Let me stress the importance of head shielding With shielding of the head we had a 25 per cent mortality figure (Figure 102)

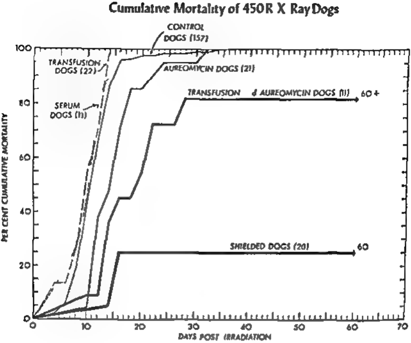
Edsall Were the shielded dogs given transfusions or aureomycin?

Allen They were given nothing

Wright Just had the head shielded?

Allen Yes

These dogs were radiated under nembutal so as to keep the head in the shield but all of our other experiments were done without nembutal



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Allen It has taken two years

Wright What about aureomycin without the blood?

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* Personal communication

of one of our studies The dotted line shows the rapidly developing thrombocytopenia Again the impairment in prothrombin utilization after irradiation can be noted The upper curve (AHF) shows that there was no change in the antihemophilic activity of the plasma it was maintained at pre irradiation levels Antihemophilic activity was measured by the assay procedure which we reported here last year (10) This is an *in vitro* test

We then proceeded to make an *in vivo* test of AHF in the irradiated dog's blood Figure 105 shows the results of one of our

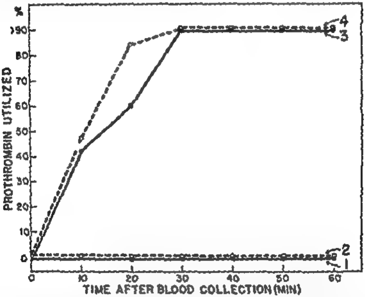

FIGURE 105 Transfusions of hemophilic dog with plasmas from irradiated and normal dogs Recipient female 21 6 kg Plasma dose 2 ml /Kg Reprinted by permission from Penick G D Cronkite E P and Brinkhous K M Plasma antihemophilic activity following total body irradiation *Proc Soc Exper Biol & Med* 78 732 (1951)

experiments in which plasma from a dog twelve days after whole body irradiation was transfused to a hemophilic dog In this experiment preliminary prothrombin utilization tests were made on both the donor and recipient immediately prior to the transfusion Curve 1 shows that no detectable utilization of prothrombin occurred in clotting blood from the irradiated donor dog Curve 2 shows similar lack of prothrombin utilization in the blood of the hemophilic dog used as the recipient Curve 3 shows what happened to the hemophilic dog's blood immediately after transfusion with the plasma

Brinkhous The material to be shown was obtained by Dr Penick and Mr Godwin of our laboratory in a collaborative study with *Dr Cronkite* * Dogs were irradiated with 600 r doses in Dr Cronkite s laboratory The studies in one dog are shown in Figure 103 Before irradiation the dog had nearly half a million platelets per cmm and the prothrombin as determined by the two stage procedure disappeared in less than an hour As the platelets decreased a concomitant impairment in prothrombin utilization was observed Finally on the ninth day no loss of prothrombin was detected in *the two hour period of study This is a confirmation of the data of* Dr Cronkite

Previously we had observed this same phenomenon in bloods made artificially thrombocytopenic by centrifugation and in hemophilia It was of interest to determine what effect this blood from the irradiated dogs at the height of the clotting defect had upon the clotting of hemophilic dog blood Figure 104 gives the results

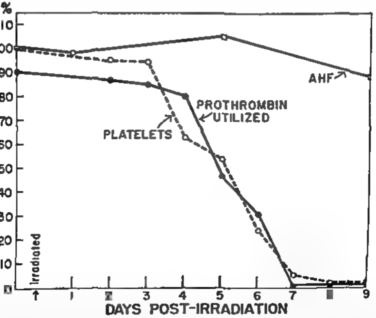

FIGURE 104 Thrombocytopenia impaired prothrombin utilization and stable plasma AHF following irradiation of 19 5 Kg male dog Prothrombin data represent prothrombin utilized in one hour after collection of blood Control platelet count 614 000/mm Nygaard method Reprinted by permission from Penick G D, Cronkite E P and Brinkhous K M Plasma antihemophilic activity following total body irradiation *Proc Soc Exper Biol & Med 78 732 (1951)*

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health U S Public Health Service

Tocantins Where in glass or silicon?

Brinkhous In glass

Alexander Under the same conditions transfusion of that dog with hemophilic blood would have done nothing?

Brinkhous That is right Hemophilic plasma similarly prepared transfused to the same hemophilic dog gave results identical with that in curve 2

We concluded from this work that there was no change in AHF of the irradiated dogs blood at the height of the coagulation defect

The only other experiment that we desire to report on this group of dogs deals with the rate of thrombin evolution The thrombin tide of recalcified citrated plasma from an irradiated dog was compared with that of normal plasma using normal washed platelets as thromboplastin The results are shown in Figure 106 Thrombin

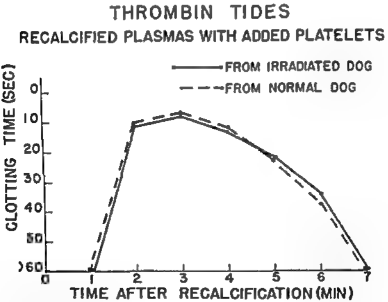

FIGURE 106 Citrated plasmas optimally recalcified Irradiated dog plasma prepared on twelfth post irradiation day

was measured by the speed of clotting of fibrinogen added to the plasma platelet mixtures at the times indicated on the abscissa Identical results were obtained with the two plasmas The irradiated plasma appears to be just as reactive as normal plasma in this test

from the irradiated dog Utilization of prothrombin was prompt Then three weeks later after the disappearance of the effect of this transfusion we transfused the same dog with normal plasma (curve 4) The minor discrepancy between curves 3 and 4 at 20 minutes probably is due to nothing more than the variability of the method

Edsall At what time after blood collection did clotting occur in those four curves?

Brinkhous The hemophilic blood ordinarily clots in about one hours The blood from the irradiated dogs clots in from 15 minutes to about 1 hour In this case it was 33 minutes The hemophilic dog blood after the transfusions clotted in nearly the same time as normal dog blood

Edsall That means one hour after blood collection

Brinkhous You are referring to the slowly clotting bloods Dr Edsall as in curves 1 and 2? The blood may clot or it may not during the 60 minute period in which periodic prothrombin determinations are being done Roughly only 1 per cent or less of the total prothrombin in blood is used in the first formation of fibrin We do our clotting times by the old Lee White method At the time we record as the end point the fibrinogen is far from being completely clotted out of the plasma I believe that these facts plus the inherent error of about 5 per cent in the two stage plasma prothrombin test accounts for our observations Thus even though the blood clotted in curve 1 in about a half hour no loss in prothrombin was detected at the end of the one hour test period

Tocantins May I ask a question about curve 3? I interpreted that as being the prothrombin utilization in a hemophilic dog after the dog had received plasma from an irradiated canine is that right?

Brinkhous That is right

Tocantins How long was the irradiated dogs plasma standing before it was given to the hemophilic dog?

Brinkhous This plasma was prepared by the silicon technique at low temperature with citrate It required nearly an hour to centrifuge and manipulate the blood prior to transfusion Precautions were taken to prevent contact with glass or to allow the temperature to go above 5 C until just prior to transfusion Also an attempt was made to keep the air interface at a minimum

Tocantins What was the clotting time of the plasma of the irradiated canine before it was put into the hemophilic dog?

Brinkhous It did not clot in the 30 minute observation period

ANTITHROMBOPLASTIN ACTIVITY OF THE PLASMA OF ANIMALS EXPOSED TO IONIZING RADIATIONS*

L M TOCANTINS
Department of Medicine
Jefferson Medical College and Hospital

I SHOULD LIKE TO stress one point that was brought up by Dr Cronkite and Dr Allen namely that in the hemorrhagic disease following exposure to ionizing radiations we are dealing funda mentally with a complex defect in the mechanism of hemostasis More than one of the various components extravascular vascular and intravascular of this mechanism are involved (1) One of the defects but not the only one is in the coagulation of blood

But is there indeed an altered coagulation of the blood in the hemorrhagic disease following exposure to ionizing radiations? We believe so There is of course a well known diminution in the number of platelets This in itself interferes with the rate of blood coagulation and with the quality of the formed clot But aside from the thrombocytopenia there seems to exist a defect in the plasma which helps to aggravate the thrombocytopenia further The change in the plasma responsible for its greater stability and slower rate of clotting resembles that of hemophilia However at least in the samples of plasma from irradiated dogs that we have examined we seldom found antithromboplastin values of a magnitude comparable to those observed in the blood of patients with moderate grades of hemophilia

Before presenting what is simply a preliminary report of our studies I shall try to outline the methods used in this work which was done in collaboration with Dr Eugene P Cronkite of the Naval Medical Research Institute Bethesda Maryland Under Dr Cronkite s supervision dogs were exposed to various dosages of roentgen rays and at certain intervals before and after exposure venous blood was collected from them into a siliconized syringe containing 19 per cent sodium citrate (1 part of citrate solution to

* The work presented in this paper was done in collaboration with R T Carroll and R R Holburn

Flynn That 10 second clot in Figure 106 means you had about two units of thrombin in your final clotting tube?

Brinkhous Yes

Flynn And it was diluted?

Brinkhous Yes the original plasma was diluted with the addition of platelets calcium the original anticoagulant citrate and finally, the added fibrinogen The final plasma concentration was about 15 per cent This compares with Dr Tocantins calculations for the plasma dilution in the one stage prothrombin time test which I believe is about 20 per cent

Tocantins Twenty per cent is correct

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ence 3) An assay of normal canine plasma was done with each assay of plasma from an irradiated dog Results were expressed either in terms of number of antithromboplastin units found per *milliliter of plasma or in per cent of normal antithromboplastin activity*

The mean of 29 assays in plasma from 17 normal dogs was 1 07 units per milliliter of plasma (stand dev ± 0 28) the range (2 × stand dev on either side of the mean) was 0 50 to 1 63 Forty six assays were done on the plasma of 9 dogs after irradiation

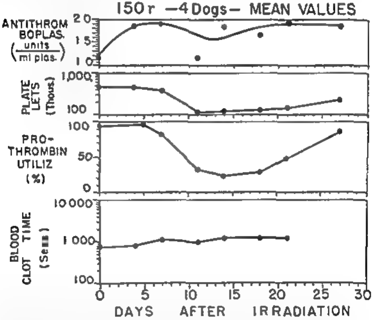


Figure 107 *Changes in the blood platelets prothrombin utilization clotting time* (glass tubes) and plasma antithromboplastin of 4 dogs exposed to 150 r total body irradiation. Note that the platelets did not go below 100 000 in any of the animals

Figures 107 108 and 109 illustrate the changes in the blood and the amount of antithromboplastin extracted from the plasma of dogs who received respectively 150 300 and 600 r of roentgen ray exposure On the fifth postirradiation day there was a slight increase in the antithromboplastin activity in all the animals This increase became more manifest at about the twelfth day when it sometimes reached approximately twice the normal value The values did not change significantly for the following four weeks

49 parts of blood) The plasma was separated by centrifugation in the cold at 3000 r p m for 1 hour Only the upper three fourths of the plasma column was removed placed in siliconized tubes stored overnight at 5 C and shipped packed in crushed ice to Philadelphia on the following morning The plasma was tested and processed immediately after arrival No opportunity was given for the plasma to freeze since freezing and thawing interferes with the stability of the plasma and its antithromboplastin activity In some of the animals tests of prothrombin utilization platelet counts and venous blood clotting times (glass tubes) were done in Dr Cronkite s laboratory With each specimen of plasma from an irradiated animal a specimen of normal plasma collected simultaneously was shipped under identical conditions These normal plasmas were tested and processed along with the plasma of the irradiated animals The following studies were done (a) response of the plasmas to simple dilution with 0 85 per cent NaCl (2) (b) clot delaying effect of adding abnormal plasma to normal canine plasmas and to hemophilic human plasmas (c) assay of antithromboplastin activity by a modification of the method reported here in 1949 (3) This modification consists in drying the plasma by suspending it in a long cellophane tubing held hammock fashion before an electric fan Twenty milliliters of plasma can be dried in this manner in about five hours The plasma and the cellophane is then cut into small pieces and macerated with forty times its original volume of absolute methanol After extraction for five days at 5 C the methanol is decanted filtered and distilled off at 40 C The residue is recovered with ether the ether is evaporated off and the residue weighed and tested One per cent suspensions in 0 85 per cent NaCl are prepared with a hand homogenizer then exposed to an ultrasonic vibrator for 15 minutes The inhibiting activity of the resulting clear suspension is tested in an activated clotting system made up of 0 1 ml lipid inhibitor suspension 0 1 ml canine brain thromboplastin 0 1 ml canine plasma 0 1 ml 0 03 M $CaCl_2$ The antithromboplastin activity of the plasma extract is compared with that of a standard lipid inhibitor prepared from acetone dried canine brain by a method described elsewhere (3) A sample of antithromboplastin prepared from canine brain arbitrarily considered to have 20 units per milligram was employed as a standard of reference A curve of activity for this standard canine brain antithromboplastin was constructed at each testing of the unknown sample, the activity of these samples being then compared with that of the standard (For further details consult refer

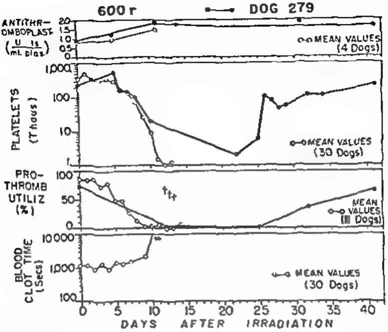


FIGURE 109 Changes in the number of platelets prothrombin utilization clotting time (glass tubes) and plasma antithromboplastin in animals exposed to 600 r total body irradiation. Figures for platelets clotting time and prothrombin utilization for this as well as for the 150 and 300 r charts were supplied by Dr Cronkite With the exception of dog 279 all the dogs receiving 600 r succumbed on or before the fourteenth day postirradiation

Tocantins We did not do that You mean assaying antithromboplastic activity before and after addition of platelets to the blood?

Alexander Yes on a sample of blood such as was used in Figure 109

Tocantins I do not know We have added increasing amounts of platelets to four plasmas with different antithromboplastin activity (from 10 to 1 unit per ml of plasma) and prothrombin conversion can be accelerated

Alexander But the antithromboplastin activity would remain constant?

Tocantins That I do not know we have not done that

Figure 112 illustrates the effect on the clotting time of diluting blood collected from an animal 13 days after exposure to 600 r The rate of clotting was markedly shortened by dilution to the level at which normal canine blood ordinarily clots

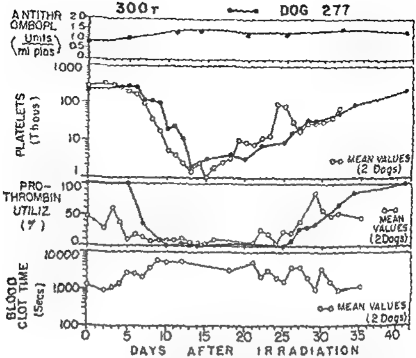
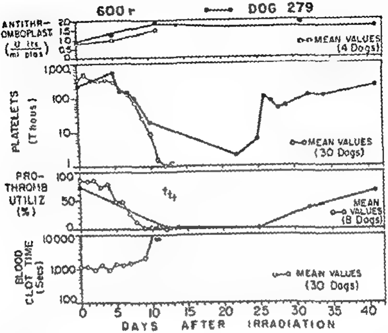


FIGURE 108 Changes in the number of platelets prothrombin utilization clotting time (glass tubes) and plasma antithromboplastin in dogs exposed to 300 r total body irradiation

The height of increase in antithromboplastin coincided with the prolongation of the clotting time and the diminution in platelet count But in view of the small number of animals employed the correlations may be looked upon as only tentative

In Figures 110 and 111 the values for antithromboplastin are expressed in terms of per cent of normal of those found in normal canine plasma simultaneously collected and examined The plasma of animals who received 150 r yielded antithromboplastin values higher than normal between the eighth and twenty first day after exposure The activity was still high the fortieth day following irradiation in two animals one of which received 300 r and the other 600 r the latter being the only animal that survived of a group that received 600 r So far it must be admitted that the evidence for the presence of an excess of antithromboplastin is not too convincing

Alexander If platelets are added to a blood showing antithromboplastic activity which would rectify the prothrombin consumption what happens to the assay of antithromboplastic activity?



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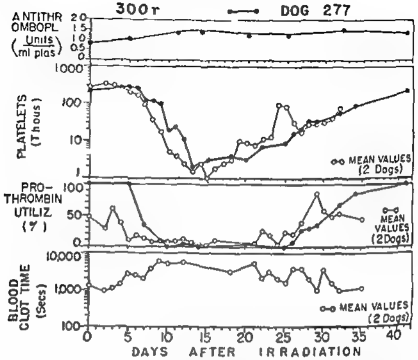

FIGURE 108 Changes in the number of platelets prothrombin utilization clotting time (glass tubes) and plasma antithromboplastin in dogs exposed to 300 r total body irradiation

The height of increase in antithromboplastin coincided with the prolongation of the clotting time and the diminution in platelet count But in view of the small number of animals employed the correlations may be looked upon as only tentative

In Figures 110 and 111 the values for antithromboplastin are expressed in terms of per cent of normal of those found in normal canine plasma simultaneously collected and examined The plasma of animals who received 150 r yielded antithromboplastin values higher than normal between the eighth and twenty first day after exposure The activity was still high the fortieth day following irradiation in two animals one of which received 300 r and the other 600 r the latter being the only animal that survived of a group that received 600 r So far it must be admitted that the evidence for the presence of an excess of antithromboplastin is not too convincing

Alexander If platelets are added to a blood showing antithrombo plastic activity which would rectify the prothrombin consumption what happens to the assay of antithromboplastic activity?

Cronkite There is one other thing that will do this in addition to dilution and that is a stream of air bubbles or agitation of the blood

Tocantins Oh yes hemophilic blood will do the same thing If shaken vigorously if air is bubbled through it or if it is brought into contact with glass surfaces or asbestos hemophilic blood will clot rapidly When normal or irradiated dog plasmas were diluted according to techniques described elsewhere (2) the resulting dilution curve resembled very much those of normal and hemophilic human plasma similarly diluted (Figure 113)

What happens if normal human or normal canine plasma is mixed with plasma from irradiated dogs? As one steps up the concentration of irradiated dog plasma and reduces the concentration of the normal human plasma the clotting times begin to rise until 100 per cent concentration of plasma from irradiated dogs is reached In fact with 100 per cent plasma clotting does not occur under 100 000 seconds (Figure 114 [A]) But even at a concentration of 50 per cent irradiated dog plasma and 50 per cent normal human plasma the clotting time of the mixture is over four times that of 100 per cent normal human plasma

Brinkhous Have you determined the residual prothrombin at intervals such as at 12 or 24 hours in this type of mixture?

Tocantins No we have not done that

When we take irradiated dog plasma and add it to normal canine plasma approximately the same sort of thing is observed as when plasma from irradiated dogs is added to normal human plasma but the clotting times are much longer as one might anticipate since the normal canine plasma was to begin with much stabler than the normal human plasma (Figure 114 [B]) But even in a mixture of 30 per cent of irradiated dog plasma and 70 per cent of normal canine plasma the mixture takes over 100 000 seconds to clot

Alexander Dr Tocantins what is the clotting time of normal canine plasma when mixed with normal human plasma?

Tocantins Unfortunately we did not do that so we do not know the clotting time

When hemophilic human plasma is added to normal canine plasma (Figure 115 [A]) a definite clot delaying effect follows until when a concentration of approximately 30 per cent hemophilic human plasma to 70 per cent of normal canine plasma is reached the mixture is incoagulable If hemophilic human plasma is added

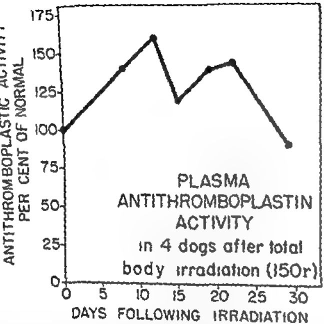

FIGURE 110 Since specimens of plasma from normal dogs were collected simultaneously with those collected from irradiated animals and the antithromboplastin activity of both plasmas was assayed in parallel fashion it is possible to express the values in the irradiated animals in terms of the per cent of those found in the normal animals

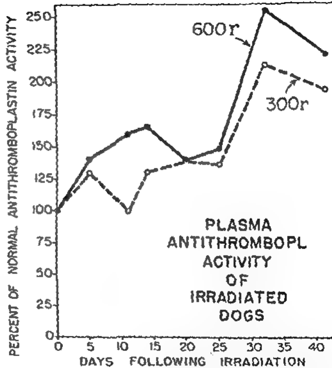

FIGURE 111 The values charted represent those in two irradiated dogs expressed in terms of those found in normal dogs

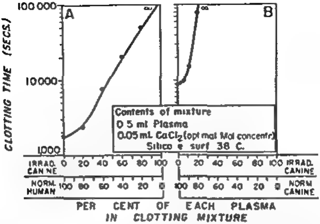

FIGURE 114 (A and B) Effect of mixing normal human and canine plasma with plasma from irradiated dogs The irradiated canine plasma clearly has a clot decelerating action on normal human plasma It has an even greater clot delaying effect on normal canine plasma

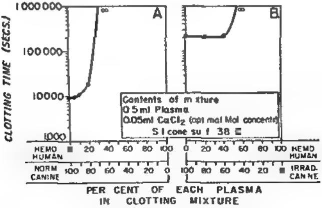

FIGURE 115 (A and B) Effect of mixing hemophilic human plasma with plasma from normal or irradiated dogs Three parts of hemophilic human plasma delay significantly the rate of clotting of seven parts of normal canine plasma The clotting rate of irradiated canine plasma is significantly delayed only when an approximately equal amount of hemophilic human plasma is added

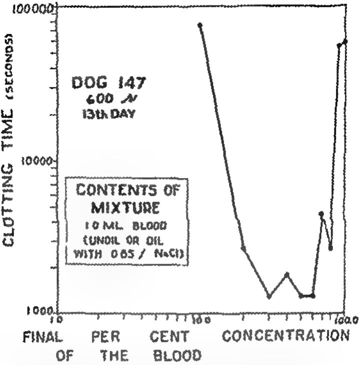

FIGURE 112 Effect of simple dilution (0.85% NaCl) on the rate of coagulation of the blood of an irradiated dog (data supplied by Dr Eugene Cronkite)

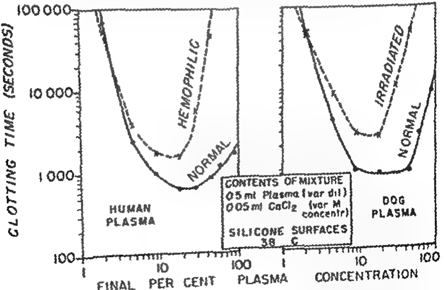

FIGURE 113 Effect of dilution (0.85% Na Cl) on normal and abnormal human and dog plasmas. Hemophilic plasma obtained from patients with hereditary hemophilia. "Irradiated" designates plasma from a dog on the twelfth day postirradiation (600 r)

because we have never assayed the blood of hemophilic dogs for antithromboplastin activity As shown in Figure 115 [A] (reading the chart from right to left) the addition of irradiated canine plasma (6 parts irradiated canine plasma to 4 parts of hemophilic human) does shorten the coagulation of the hemophilic human plasma

In summary therefore it seems that in the blood of irradiated dogs there are two defects to account for the rate of delayed coagulation The principal defect is a very marked thrombocytopenia but in addition to that and at about the time the thrombocytopenia is most pronounced there is a slight increase in antithromboplastin in the plasma This increase by itself and in the absence of a severe thrombocytopenia probably makes little difference in the rate of coagulation of the blood especially in glass tubes (see Figure 107) But the thrombocytopenia is helped and in a sense aggravated by the slight increase in the antithromboplastin and the end result is a greater stability of the blood and a prolongation of the clotting time Neither one of these changes by itself would probably result in more than a slight delay in the clotting time When experimental thrombocytopenic purpura is produced in dogs by means of antiplatelet serum (5) the rate of coagulation of blood (paraffin tubes) is only slightly delayed even during the acute phase of the thrombocytopenia If at this stage the antithromboplastin content of the blood were increased the blood might well become hypo or incoagulable Experiments to duplicate these conditions are now under way in our laboratories Lipid antithromboplastin extracted from canine brains is injected intravenously and the dose adjusted so that it may reach a concentration in the plasma like that found in the plasma of irradiated dogs Measurements of the rate of coagulation of the blood can then be made and compared with that of the blood of normal animals that have received the same amount of lipid antithromboplastin intravenously

Wright Dr Best have you any comments?

Best Just a short one about some work that Dr Monkhouse and Dr Fidlar (6) are going to publish on heparin content of blood from irradiated animals The new method of Dr Monkhouse detects less heparin than the minimal required to get a perceptible change in clotting time (that is it is more than adequate for the purpose) With this method Dr Monkhouse does not find any heparin liberated Dr Allen referred to that already But recently Dr

to plasma from irradiated dogs the hemophilic human plasma can prolong the clotting time of even the plasma from irradiated dogs A mixture made up of 50 per cent hemophilic human plasma and 50 per cent irradiated dog plasma does not clot under 1 000 000 seconds and significantly delays the clotting of plasma from irradiated dogs (Figure 115 [B])

In connection with these plasma mixture experiments it is well always to keep in mind that interpretation as to the existence of a clot accelerating or clot delaying effect depends on the point of view of the observer Experiments of this type cannot be taken as absolute demonstrations of the existence of a clot inhibiting or clot accelerating effect unless one adheres to a minimum standard of performance for this effect such as that one part or less of the plasma tested must delay the clotting of three parts or more of normal plasma by at least one third the clotting time of an equal volume of whole normal plasma (4) Conversely a clot accelerating effect should not be held to exist unless one part of the clot accelerating plasma shortens the clotting time of three parts or more of the stable plasma by at least one third the clotting time of an equal volume of whole stable plasma When the stable plasma is incoagulable these conditions are not possible to fulfill Without such standards as a guide it is difficult to be sure in a given plasma mixture (e g Figure 114 [A]) whether the addition of normal human plasma shortened the coagulation of the irradiated canine plasma (reading the chart from right to left) or whether the addition of irradiated canine plasma prolonged the coagulation of normal human plasma (reading the chart from left to right)

The data so far presented are obviously still of a fragmentary and incomplete nature The assay studies leave us only with an impression that there is an increase perhaps a two fold increase in the plasma antithromboplastin of irradiated dogs This impression plus the results of the dilution studies seems to indicate that the range of the defect in this plasma lies between hemophilic human plasma and normal canine plasma It is possible to recover of course much more antithromboplastin from hemophilic human plasma than from irradiated dog plasma This may explain why in Dr Brinkhous experiments the injection of irradiated dog plasma into the hemophilic dogs accelerated the coagulation and prothrombin utilization of the blood of hemophilic dogs perhaps because there is much less antithromboplastin activity in the irradiated dog plasma than in the hemophilic human plasma and by analogy in the hemophilic canine plasma I say by analogy

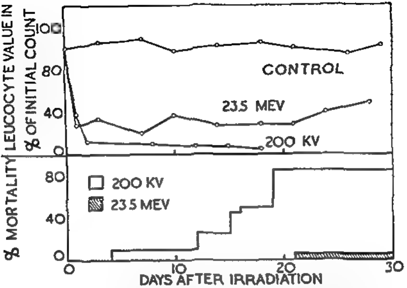

FIGURE 118 Leukocyte value and mortality of mice after radiation with betatron and 200 kv radiation C57 Mice average age of 112 days Radiation dosage was 600 r for both types of radiation as determined by 100 r Victoreen chamber

tality until the third week at which time there was a small mortality The chief interest of this is the fact that the mortality as judged by inspection of the animals was by hemorrhage whereas the mortality of the mice exposed to the 200 kv was not related to hemorrhage it was undoubtedly related to the leukopenia This study while complete for another purpose is just the beginning of work as far as hemorrhagic symptoms are concerned It does suggest that different types of radiation might give a simpler technical approach to the problem Of course with further study we will have to see whether the underlying hemorrhage has the same cause with different types of radiation

Wright Very interesting Any questions?

Cronkite Dr Jaques brought up a very good point with which I know Dr Allen agrees and to which I hope Dr Edsall does not take exception The biological response in terms of the various parameters that can be measured such as mortality rate survival time leukocyte levels splenic thymic weight decrease etc is a more accurate and reproducible index of the absorption of energy by the tissues than the measured ionization in air Because of self absorption by various sized animals and the scattered radiation from the surround

Monkhouse and Dr Fidlar sensitized three different species of animals and then irradiated them looked for heparin in their blood and found none then they gave the animals antigen and found a great deal of heparin in all three of the species In other words the irradiation did not desensitize and they got the liberation of heparin proving that heparin was available but none was liberated by irradiation That is a little further proof of the same thing you have just said Dr Allen

Allen May I ask if those animals showed clinically the degree of anaphylactic manifestations that one sees in nonirradiated animals?

Best I could not state the exact extent

Allen It would take a lot of animals to do that

Best There were gross signs of anaphylaxis but whether they were of the same magnitude I don t know

Brinkhous What antigen was used?

Best Alum precipitated horse serum

Jaques I want to underline some of Dr Cronkite s remarks about the question of the type of radiation and the type of species because I think there is a great danger in talking about the hemorrhage in irradiation sickness as though there is just one symptom and one cause which is probably not the case if one takes all types of radiation

Dr Cronkite s results were obtained I gather chiefly with a 2 mev machine We have a betatron which gives 23 5 mev and for the last two and a half years we have been comparing the effects of 23 5 mev radiation with the ordinary conventional x rays * Observations of hemorrhagic symptoms have been incidental to other studies Figure 116 shows a comparison of a dose of 600 r on leukocyte counts in mice The upper curve is for control animals The second curve is for 600 r from the betatron (23 5 mev) and the third curve 600 r from the conventional 200 kv x ray The mice were placed in the beam in a rotor so that there was a uniform radiation of the body within 4 per cent

The point that I think is of some significance here is that with 200 kv the leukocyte value fell to a very low value and as a result there was an increasing mortality in the mice which is shown in the lower rectangle The accelerated mortality eventually reached over 80 per cent on the 200 kv On the other hand with the betatron the leukocyte values fell much less and there was no mor

* Data supplied by Dr T P Ting To be published under the authorship of T P Ting H E Johns and L B Jaques from the Departments of Physiology and Physics University of Saskatchewan and the Saskatchewan Cancer Commission

PLATELET TRANSFUSION

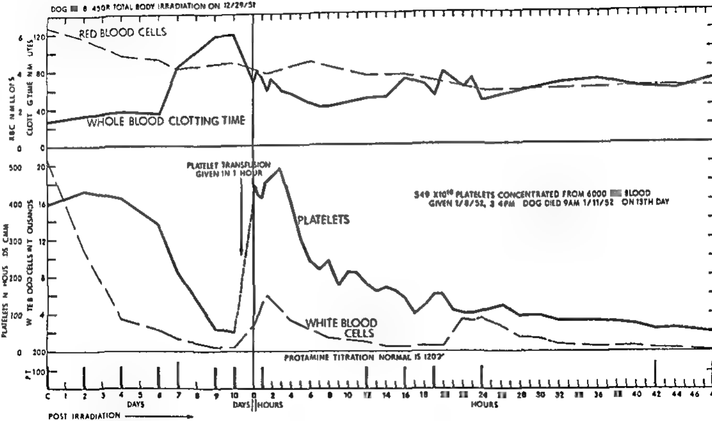

FIGURE 117

ing medium and from the tissues the air dose does not accurately reflect energy absorbed unless the animal is free in air and the radiation is of high energy In other words the distribution of energy to sensitive tissues and the total energy absorbed are of equal importance There is no way to quantitate accurately the distribution of absorbed energy in tissues with varying energies of radiation and varying sizes of animals Hence the gram roentgen is of limited value in whole body exposure because for example only a few milligrams of mouse spleen need be shielded to double the mid lethal dose The measurement of the whole body exposure is a complicated problem The roentgen unit is not comparable to a milligram of an injected drug

Allen It is well to point out here that Dr Cronkite s 600 r is biologically equivalent to our 450

Cronkite With one little difference that I noticed today and never noticed before

Allen What?

Cronkite That your platelet levels do not go as low as ours

Allen They go to the point where one is counting dust They are at that level where one could argue over whether we are counting anything With phase microscopy perhaps that is different but I don t think there is a significant difference because biologically your mean survival time is the same It was 11 2 or 4 and so is mine

Cronkite There is another mathematical consideration I should like to bring up I am not a mathematician I don t know how to approach it You give 150 r and the pattern of the decrease of the platelets or leukocytes is rather reproducible It goes to a constant level that is more or less a function of the dose of radiation that has been given If any of the things that have been put in the literature about life span of formed elements are correct production of these cells must be going on or they would disappear as they do when one gives high enough doses of radiation How does one decide what the rate of production is? Is the production rate normal or impaired? If it is impaired and platelets for example are being needed why does a constant lower level occur? Can this be explained by impaired utilization? I just cannot think the thing through myself Something is being produced at a slower rate and if consumption remains unchanged the circulating level should progessively decrease not reach a steady state

Allen But isn t that true of all biological phenomenona that there is production utilization or destruction whatever you wish to call it? It is true of all the clotting factors

matology Extensive routine hemocytologic and histopathologic (autopsy) examinations were conducted but I shall have little to say about those in the present report What concerns us here are the special blood clotting studies particularly the prothrombin consumption tests (*a*) by the two-stage method and (*b*) by the modified one stage method Some other procedures will be described also

Control tests were run on all the dogs prior to the gold injections and the one stage prothrombin consumption tests are charted in Figure 118 Two additional animals after preliminary testing

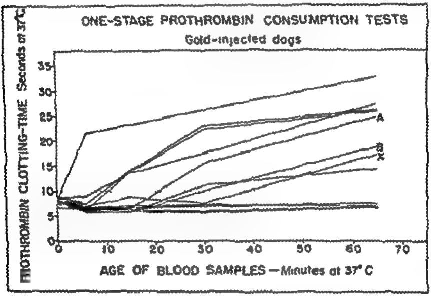

FIGURE 118

received 6 ml * of *stable* colloidal gold intravenously and were subsequently tested repeatedly over two weeks According to our tests these control animals remained perfectly normal

Figure 119 shows effects on the prothrombin consumption tests at the sixth day after the gold injection These prothrombin determinations were made by the *two* stage method on oxalated plasma (0 minutes) and on the serums removed after oxalation of consecutive blood samples aged respectively for 15 minutes 30 minutes 60 minutes plus clotting time and 90 minutes at 37 C Values are recorded as the percentage of the original plasma unitage The

* Volume comparable on per kilo basis to that used in the two animals given 10 mc per kilo of radiogold

I want to show what happens when the platelets go up to 460 000 inside of 24 hours Counting every two hours they are right back to where they were at the start at 20 000 or 30 000 in another 24 hours (Figure 117)

Wright Perhaps Dr Edsall has an idea on this

Edsall I am afraid I don t I might say that although I have had no experience with radiation work in animals myself I agree with Dr Cronkite s general point of view It seems to me that the biological response is the important thing and that our present physical measurements are often inadequate to indicate what is really happening in the animal with a given physical dose But as to the matter of maintaining a constant level I don t know

Ferry Any dynamic equilibrium is a balance between a forward reaction and a backward reaction and the level reached is deter mined by the relative rates of those two reactions

Allen That must vary though because a patient with liver dis ease who is deficient in prothrombin is in a relatively stable situa tion since he may maintain his prothrombin let us say at 45 per cent of activity for weeks Obviously his destruction and production of prothrombin are in equilibrium at a different level from what they were when he was well

Cronkite When dogs are irradiated with 150 r the mean platelet count falls to 100 000 per mm^3 and remains at a relatively constant level for 2 to 3 weeks One assumes that platelet production is impaired because of the bone marrow damage The animals are on the verge of bleeding and we further assume that platelets are if anything needed in larger numbers than normally If needs and consumption are normal or greater than normal and if production is impaired why is a steady state maintained in the peripheral blood? Why doesn t the platelet count continue to drop? Could the needs and consumption be decreased by radiation? It just doesn t make sense to me at the present time

Ferguson We have recently initiated a new program with the cooperation of Drs M Brucer and G A Andrews of the Oak Ridge Institute of Nuclear Studies using radioisotope injections in an attempt to localize radiation damage principally to certain specific parts of the body so that the effects on the blood coagulation mechanisms may be studied I should like to report on a series of dogs receiving intravenous injections of radiogold (Au^{198}) in the form of colloidal gold sol Five dogs received respectively single iv injections of 20 10 10 5 and 1 6 millicuries per kilo and were thereafter isolated and observed for general and particular sympto

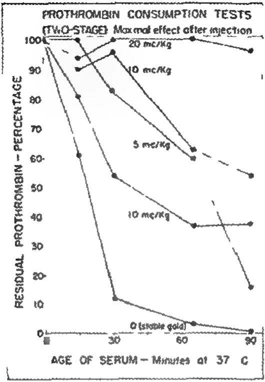

FIGURE 1..0

The course of the anomaly appearing in these tests at the various dosage levels is apparent in Figure 121 Here are charted the results of the 90 minute tests for all dogs throughout the study period We have omitted tests on a number of untreated dogs since the two animals receiving stable gold behaved quite similarly showing only traces of residual prothrombin in repeated tests over a 10 to 13 day study period One animal receiving a token dose of 1 6 millicuries per kilo is included in our series Since the residual prothrombin remained below our critical level of 10 per cent we doubt whether the slight elevation of its data is significant The tests on the dog receiving 5 millicuries per kilo became significant on the 6th day and the defect increased until the 18th day (a severe reaction) and then returned to normal (nearly) by the 29th day

In one animal receiving 10 millicuries per kilo the test became positive on the 6th day more so through the 12th day fluctuated in severity in tests at the 30th and 49th day and was back to normal

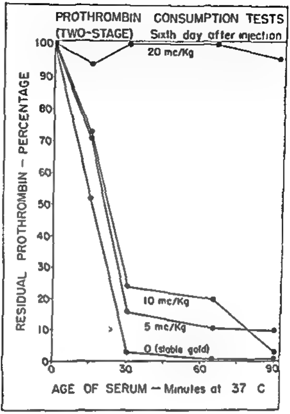

FIGURE 119

control injected with stable gold is quite normal with negligible residual prothrombin after 30 minutes Anything more than 10 per cent of residual prothrombin in the 60 to 90 minute period is to be regarded as significant evidence of impaired prothrombin utiliza tion This was consistently found by the sixth day after each of the recorded dosages of radiogold being extremely marked with the highest dose of 20 millicuries per kilo

The maximal effects noted throughout the study on four irradiated animals are similarly shown in Figure 120 Another control with stable gold is included Marked lessening of prothrombin consump tion is evident in all the irradiated animals In general the maximal effect tends to be somewhat proportional to the radiation dosage The dog receiving the 5 millicuries per kilo dose suffered dispro portionally however *

* This (11 day) test on the 5 mc per kilo dog was in fact not the maximal an even greater defect appeared in the 14 and 18 day tests which are included in Figure 121

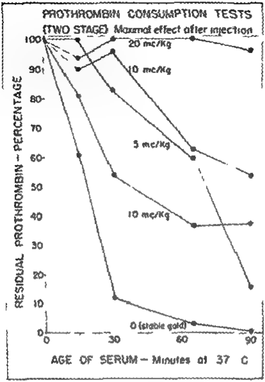

FIGURE 120

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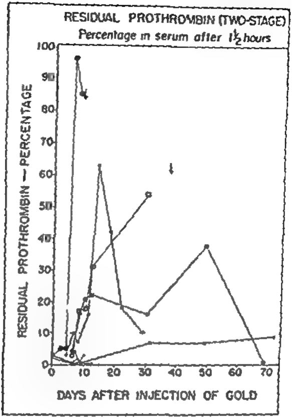


FIGURE 121

on the 69th day This animal developed marked icterus indicative of liver damage but this was only slight on the 69th day and the general condition of the animal was suggestive of recovery It died unexpectedly however on the 87th day

In the other animal receiving the 10 millicurie dose the course during the first ten days was very similar to its companion There after the prothrombin consumption test data icterus and clinical condition of the animal were more severe and progressive Death occurred on the 37th day with terminal hemorrhagic manifestations characteristic of radiation sickness together with autopsy findings of severe liver damage

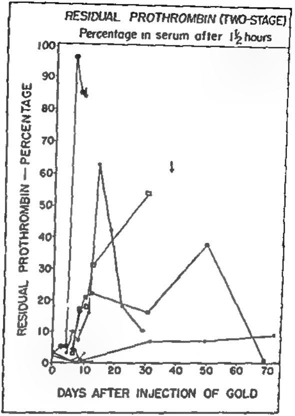
In the dog receiving 20 millicuries per kilo it was interesting to note normal test values on the 2nd and 4th days after the isotope injection On the 6th and 8th days there was severe icterus and very little utilization of prothrombin Death occurred on the 9th day with typical hemorrhagic manifestations including bleeding

into the neck from the venipuncture of the previous day This incidentally was the only obviously traumatic hemorrhage encountered in these experiments Autopsy showed another particularly interesting finding Not only was the liver markedly icteric and necrotic but a couple of inches of the duodenum just where it approximated most closely to the liver was swollen with a massive interstitial hemorrhage

The more generalized hemorrhages in the last two animals included diffuse bleeding in the lungs petechiae in serous membranes (subendocardial subepicardial subperitoneal) and spotty hemorrhages along the alimentary canal together with interstitial hemorrhages in mesentery and retroperitoneal tissues

Figure 118 summarizes the maximal effects on the prothrombin consumption tests made by the one stage method The chart records actual clotting times at 37 C on the mixtures of serum (or plasma) $BaSO_4$ plasma (to supply fibrinogen etc) thromboplastin and calcium salt The longer the clotting time the smaller the amount of residual prothrombin The dotted line at a value of 10 seconds is the critical level according to our experience which divides the data into two groups In the first or normal group this level is regularly exceeded in the 30 minute and later tests In the second or positive group significant defects in prothrombin consumption are indicated by the persistence of shorter clotting times in the 60 to 90 minute period It is readily seen that the normal group includes all animals tested before the gold injections the two animals (A and B) receiving stable gold and probably the one animal (X) receiving the token injection of 1 6 millicuries per kilo of radiogold although its 30 minute test is below that of the other normals In marked contrast are the low data lines remaining well under the 10 second critical level of the four irradiated dogs receiving between 5 and 20 millicuries per kilo

It is possible to compute percentages of residual prothrombin from the one stage clotting times of serial dilutions of normal dog plasma We have omitted presentation of this for several reasons First such reference curves are insufficiently standardized and vary somewhat from animal to animal and in the same dog from time to time Secondly the results in the irradiated animals follow the trend of the two stage prothrombin data only qualitatively and are not strictly comparable quantitatively An important possible explanation of this is contained in the third point As can readily be seen from nearly all the curves of Figure 116 the prothrombin clotting time values in the 8 15 and often 30 minute normal tests



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bomb) radiation by Cronkite Penick and others The last named added the finding that the antihemophilic factor was not affected by fatal irradiation

In our study whole blood clotting times remained normal except for a slight prolongation (about twice normal) in the 20 millicuries per kilo dog on the 6th day Clot retraction however was definitely lessened in the 6th to 8th day tests on the 20 millicuries per kilo animal and in the periods 10 to 30 days and 10 to 49 days on the two animals receiving 10 millicuries per kilo It was normal in all others

I shall not discuss the red cell and white cell changes but a few comments on our platelet data are in order It may be remembered that Cronkite Penick and others believe the thrombocytopenia to be (*a*) extremely significant in accounting for the defect of prothrombin consumption and (*b*) one of the important factors in the hemorrhagic syndrome of radiation sickness There is no doubt that the platelet counts were significantly lowered in the later stages of our more severely gold irradiated dogs Accurate platelet counts are difficult even for an experienced laboratory technician and because of the working conditions at Oak Ridge these data were not up to the highest research standards We are not prepared therefore to argue this point at all strongly but would merely like to suggest that we had some indications of a marked reduction in the earlier prothrombin consumption tests when the platelets were still close to normal limits What we do stress is that the platelets require some plasma factor to work with them in supplying the thromboplastic mechanism for normal plasma prothrombin conversion We suggest that future studies look further into this plasma component about which we know very little at this date before concluding that the prothrombin consumption defect which seems to be so typical of the effects of severe irradiation is solely due to reduction in the number or quality of the circulating blood platelets

In summary therefore internal radiation with radiogold (Au^{198}) in sufficiently large doses and after several days latency induces a blood coagulation defect of which the prothrombin consumption test is a sensitive index Our limited experience to date offers the further suggestion that this test may be of wide practical usefulness in the early detection of severe radiation injury and may perhaps prove to be sufficiently quantitative to follow the course and indicate the ultimate outcome of many types of radiation We have begun to accumulate data on human cancer patients under radia

diminish instead of increase This phenomenon has been noted by others notably by Langdell Graham and Brinkhous (7) Since the two stage prothrombin tests do not show this phenomenon it seems evident that the one stage test involves factors other than ordinary prothrombin Without entering into any discussion of this big question we may suggest that at least part of it concerns the elaboration of the spca factor (of Alexander) or convertin (of Owren) Whatever the explanation the fact is demonstrated in our present studies that the same phenomenon occurs in the serums of the gold irradiated animals Indeed, owing to the persistence of low prothrombin clotting time values it is commonly found even in later tests

On several occasions we added a simple test which we have previously found useful for detecting circulating anticoagulant (heparin antithrombin for instance) and also a variety of deficiencies in the blood clotting system Simple recalcification clotting times at 37 C are noted for varying mixtures of test plasma and normal dog plasma Table XVIII illustrates this test on plasmas of

TABLE XVIII

Test for Circulating Anticoagulant, etc Recalcification Clotting Times, Seconds at 37 C

% Test Plasma	0	25	50	75	90	95	100
Dog 204 (10 d)	55"	58"	57"	56 3"	61"	59"	63"
Dog 204 (12 d)	123"	73 5"	62 5"	63 8"	60 3"	62	59 3

one of the dogs 10 and 12 days after receiving 10 millicuries per kilo of radiogold The 0 column is the normal plasma alone and the 100 column the unmixed test plasma The absence of any significant increase in the clotting times of the various percentage mixtures rules out the anomalies mentioned In the 12 day experiment another phenomenon is incidentally noted Here the normal plasma was giving unduly prolonged clotting time (123 seconds) attributable to loss of labile accelerator factor (AcG or accelerin) from two day storage in the icebox The test plasma additions correct this This is crude but presumptive evidence that the plasma of irradiated dogs contains an adequate amount of accelerin (AcG or labile factor)

Our tests on internally irradiated dogs substantially confirm the clotting studies on animals exposed to external (x ray and atomic

TABLE XIX

Comparison of Platelet Counts (A)* and Residual Prothrombin (B)† in Dogs Injected with Radiogold

Day After Injection	DOSAGE OF RADIOGOLD					
	20 mc /kilo		10 mc /kilo		5 mc /kilo	
	(A	(B)	(A)	(B)	(A)	(B)
0	354 500	4%	238 400	–	312 500	–
2	217 500	7%	765 000	–	–	–
4	242,500	5%	–	–	337 500	5%
6	45 000	100%	339 650	20%	372 500	11%
8	22 500	667	139 400	41%	135 000	3%
10	(died 9d)		219 000	27%	–	60%
13			26 000	83%	77 500	67%
18			13 100	65%	145 000	34%

* (A) Platelet counts per cu mm
† (B) Residual prothrombin percentage left in 60 min + clotting time by two-stage test

penia is produced Then it is followed of course by a slow return to the normal level

Ferguson Did you do the prothrombin consumption tests at that time?

Tocantins We did not do those We simply followed the platelet count In fact we have used radioactive gold as a method for bringing down the number of platelets in patients with thrombocytosis who are having repeated thrombosis One chap who had a splenectomy was having repeated thrombosis and we brought his platelet count down by injecting radioactive colloidal gold

Alexander May I make one remark about this accelerated prothrombin time shortly after blood is withdrawn? Dr Brinkhous reported that that occurs in dogs and we have found it also in humans It has also been reported by Quick We can now state unequivocally that it is due to spca rather than to the conversion of plasma Ac globulin into the serum type because it also happens in parahemophilia (8) in which Ac globulin is absent or markedly deficient It is a phenomenon that takes place early in the course of spontaneous coagulation

tion therapy in order to learn more about this interesting phe nomenon

Seegers Dr Ferguson you say you have inadequate platelet methods?

Ferguson I am critical of them

Seegers Are you going to improve them?

Ferguson I hope to I have been doing the clotting studies and have relied upon the ORINS laboratory for the hematological data They have a good technician who does the routine hospital work She has been using a clinical routine method of counting platelets and red cells on coverslip films and estimating platelets from the ratio and the regular erythrocyte count A week or two before our studies she tried to master the pipetting chamber phase microscope technique used by Dr Cronkite She did not feel that she had achieved sufficient proficiency with this method and it was not practical to set it up in the small building out in the area where the dogs were kept The films on the other hand could be brought into the hospital laboratory and stained and examined between the pressures of her regular routine Hence I am inclined to regard our platelet counts as reasonably accurate by clinical rather than by research standards Having stated these reservations I am repro ducing the data from three animals respectively receiving 20 10 and 5 millicuries per kilo of radiogold The residual prothrombin in the one hour serum samples as measured by the two stage method and recorded as percentage of the original plasma unitage is included for comparison in Table XIX

There is no question that when the platelet counts are really low in the later tests the prothrombin utilization is also much reduced This agrees with Cronkite s and Penick s data on external whole body irradiation In the 6 day tests on the 10 and 5 millicuries per kilo dogs however the platelet counts are normal but the prothrom bin consumption is significantly lowered Again on the eighth day in these two dogs platelet counts are lowered to similar levels but the results of the prothrombin tests are widely different We shall need a much more extensive series of data than is immediately in prospect before we can feel confident of these results

Tocantins We have used radioactive colloidal gold in the treat ment of hemolytic anemias particularly We have been impressed by the fact that it is a thrombocytopenia inducing agent Within approximately two weeks after the injection of radioactive colloidal gold intravenously in doses of about 10 to 40 millicuries that is 1/5 to 1 millicurie per kilo body weight in adults a thrombocyto

lipoprotein in any quantity It occurs in the S_f 3 12 range and it is present only to the extent of 20 to 30 mg per cent It is difficult to disturb this lipoprotein picture in the rabbit It cannot be done by feeding the rabbit large quantities of food and a disturbance by cholesterol feeding requires several days

The rabbits that subsequently die after radiation are those which develop this opalescence within the first twenty four hours due to large quantities of lipoprotein material above S_f Most of them tend to be in the molecular range of S_f 30 to S_f 200 Depending upon the stock of rabbits a lipoprotein conversion block may occur anywhere in the range of S_f 20 to 200 Lipemia is observed during the first two days Then rapidly thereafter the atypical S_f 10 200 lipoproteins disappear and with thus disappearance a great increase of the normal lipoprotein molecules is seen as though the S_f 20 200 material were transformed to S_f 3 10 lipoprotein The normal lipo protein may at this time increase by a factor of tenfold or more

The strongest evidence for a postirradiation heparinemia is the appearance at the third day of detectable active factor material in these irradiated rabbits This is at the same time that the induced S_f 20 200 molecules are disappearing rapidly and being replaced by S_f lipoproteins which are normal to the rabbit We don t know whether this lipoprotein converting active" factor produced on the third day is caused by free heparin or not At least it is similar in action to the substance that can be generated by the administra tion of heparin to an animal Neither can this be given as evidence for free circulating heparin in these animals Extremely small quan tities of heparin will generate the lipoprotein conversion active" factor In fact we have used this to try to test for absorbed heparin in the sublingual type experiments without seeing any evidence of absorbed heparin

Cronkite In reference to Dr Allen s comment about the relative importance of hemorrhage and infection as a cause of death Pro fessor J A Reyniers and his group at the University of Notre Dame are studying radiation injury in the germ free rat These studies will eventually elucidate many of the problems about the relative importance of infection hemorrhage and anemia as causes of death in the irradiated animal

Wright That should be an important study

Cronkite Yes the studies should be critical At the present time we don t know what to think So far we have not been able to control infection in dogs with antibiotics Infection appears to be the major cause of death in our experience so far Certainly if

Allen I get the idea from this discussion that prothrombin conversion is a rather specific reaction due to but one defect If that is the intention I don t agree with it I would doubt whether that is correct and whether other people would feel the same way about it I should like to hear a little discussion

Ferguson The point should not be made that it is just one defect namely a platelet defect until other possibilities have been fully explored

Allen If there is anything that will inhibit the first phase of coagulation it delays the conversion of prothrombin

Tocantins There are at least four factors the two accelerators and in addition the platelets and the first phase inhibitors

Seegers And through that you are making the assumption that you have not done anything to prothrombin?

Tocantins That is right Thrombocytopenia diminution in accelerator factors platelet accelerators as well as the plasma accelerator globulin excess of first phase inhibitors all these factors will influence the rate of prothrombin conversion Diminution in the rate of prothrombin conversion is not a defect due to a specific cause It can result from a number of causes

Allen There is one other thing that needs an expression of opinion and that is to what extent does this hemorrhagic picture of irradiation contribute to the mortality of the animals? It is my own belief that while hemorrhage is the most spectacular finding at autopsy and undoubtedly many times is the cause of death even if hemorrhage were not present the animals would continue to die So that while we were originally impressed with the severity of the problem of hemorrhage as a cause of death I would certainly say that it is a contributory factor but that its control will not play a very important role in determining whether or not the animal will survive

Jones We have some observations on the irradiated rabbit that may blend into some of these other observations fairly well The rabbit is an unfortunate animal to compare with the dog because it does not produce the same hemorrhagic signs at the terminal state The rabbit is fairly sensitive to radiation in doses of 700 to 800 r and will die in about two weeks to a month after irradiation Rosenthal (9) showed that an opalescence appearing in the plasma during the first twenty four hours after irradiation was quite prognostic as to whether the particular rabbits would live or die We have investigated this lead because the opalescence seemed most likely to be a lipoprotein (10) The normal rabbit has only a single

penetration of a vital organ or severance of a large blood vessel are so clearly understood that their enumeration is superfluous In many diseases however as in Hodgkin s the conditions producing death are not so easily detectable Who can elaborate and quantitate all the conditions such as manition anemia pneumonia anoxia blood volume changes tissue replacement electrolytic disturbances and so on which eventually led to death? It is obvious that terminally the conditions in such a disease are so inextricably interwoven that our current methods seldom permit the separate threads to be followed From a practical standpoint we can only select the basic condition of a disease and formally attribute death to it realizing that many other indispensable conditions were superimposed before death resulted

In the present discussion of irradiation illness it is obvious that death cannot be assigned to a single factor Although no one will deny the merit of attempting to analyze the many conditions concerned in fatal irradiation illness it must be remembered as Dr Allen hinted that death is always the result of the combined effect of many factors and it is impossible to make one component of the aggregate the exclusive cause

Wright Amen

Cronkite I think Dr Flynn s comments are pertinent The radiation syndrome is very complex The causes of death are multiple One can only state that radiation induces a pancytopenia which makes the animals susceptible to various sequelae of the pancytopenic state any of which by various routes may result in death It still remains of interest that susceptibility to infection could not be correlated with the factors known to be connected with defenses against infection The animals were most susceptible when studies indicated they were returning to normality

Allen On the other hand if we don t find out what the causes of death are we certainly will never have a therapeutic program that we can depend on The problem does have to be unraveled

Cronkite From a clinical standpoint did we ever have to do any studies to know how to treat radiation injury?

Allen Yes

Cronkite I mean this seriously Antibiotics and blood transfusions when indicated were indicated before radiation was studied in detail and still remain the major members of the therapeutic armamentarium

Allen You may be making a mistake on the transfusions The transfusion problem is the one that has yet to be solved Certainly

animals are irradiated in the germ free state where infection is impossible and the animals bleed or die in the absence of infection control of infection by drugs will not be expected to increase the *survival rate consistently or significantly over a wide range of doses* of radiation

Allen It is important to point out that C P Miller (11) at the University of Chicago has done extensive studies on mice with regard to infection and he cannot correlate his cumulative mortality with septicemia or with the leukocyte count

Edsall *How much is known about the effect of irradiation on* various systems involved in tissue metabolism?

Cronkite A lot is known The more significant studies have been performed by DuBois and others (12) at the University of Chicago Toxicity Laboratory on alterations in citrate metabolism in liver thymus and spleen I don t care to comment in detail on these changes because of limited knowledge of the field

A really frustrating experience is the irradiation of animals with sublethal doses of radiation This has been done by Shechmeister and Bond (13) Initially I couldn t believe their results They exposed mice to sublethal doses of x ray and then challenged them with an aerosol of Streptococcus zooepidemicus The sublethally irradiated mice were much more sensitive than normal mice which might have been anticipated However the maximum susceptibility could not be correlated with any of the known changes in the granulocyte count etc It might reasonably have been expected to find maximal susceptibility when granulocytes were minimal This was not the case, but it occurred when bone marrow and peripheral counts were well in the recovery phase

Flynn This discussion as to the cause of death is a philosophical problem Legally of course death means a permanent cessation of the cardiac function but we all know that many tissues like the cornea can be successfully transplanted after death The immediate cause of legal death is in fact the totality of all the conditions which lead to a cessation of the heart beat Although each condition contributing to *the total is unable by itself to produce death* it still forms an indispensable component of the aggregate This plurality of conditions required for an effect (or what philosophers call a *consequent*) is of course the principle of multiple causation I have already referred to

While it is true that in certain instances we may assign a causal role to only one condition say a gunshot injury or stab wound we do this by virtue of the fact that the other conditions such as the

Edsall May I ask Dr Allen one thing? The injection of whole blood is the injection of an exceedingly complex system Suppose you were to inject washed red cells for example instead? Is it possible that there is something in the plasma which is actually deleterious but that the red cells without the plasma might be beneficial?

Allen We shall know that in a couple of more years We have just accumulated 50 liters of dog plasma to do the same thing with plasma alone and then we shall go back to washed cells

Cronkite I want it on the record that I personally feel very strongly that transfusions will be an essential part of the armamentarium for treatment of radiation injury

We have used transfusions in dogs but not daily transfusions which provide a chance for the animal to become sensitized to unmatched blood We have let them get down to the point where they were dyspneic and so anemic that they were ready to die Then they have been given 300 or 400 ml of whole blood and they get up and start eating again

Allen I should like to have you do the same experiment we did and see what your data would be

Cronkite You mean repeat it every day?

Allen No you don t have to do it that way but take that same number of dogs and prevent fatal anemia by our method If you come out the other way I shall certainly be willing to reconsider But on the basis of data now available no one can say that blood offers us any real hope in either the control of bleeding or in the increase of survival rate

Tocantins Blood as given in your experiments in dogs?

Allen Blood was cross matched but certainly there are better ways to cross match dog bloods where there are subtypes I might add that we found very few incompatible bloods in our cross matching in selecting donors

Cronkite Don t you mean that the maintenance of hemoglobin is not going to increase survival rate? You don t mean to infer that transfusions can be harmful if the bloods are properly matched? Do you really mean that?

Allen I would have agreed with you a few years ago but not any more We have no evidence that blood alone is beneficial in the control of hemorrhage or improvement of survival rate

Cronkite There is only one dog that I can really talk about That dog received 600 r and had just a little more than 2 Gm of hemoglobin per 100 ml

transfusion alone is of no benefit It simply enables the animal to die with a normal hemoglobin

Tocantins But that is not the experience with the same problem in man

Allen We dont have irradiation data from man to work with We dont have a correlating group

Tocantins Well there are the hypoplastic anemias with severe thrombocytopenia

Allen You are dealing there presumably with but one system and a few perhaps interrelated systems Here you have an irradiated animal in which all tissues have been exposed There are damages that we dont have any idea about I think it is a mistake to consider irradiation injury in terms of pancytopenia in man though the irradiated animal also develops pancytopenia

Tocantins But at least we might say this that blood transfusions do have a beneficial effect in the hemorrhagic disease of pancytopenia in man

Allen Yes but dont carry that over to irradiation injury until it can be justified experimentally

Tocantins Well that much can be said for it in the human being Can we say the same for the experimental animal?

Allen We cannot

Tocantins In the hemorrhagic disease of irradiation pancytopenia I am not talking about effect on simple survival

Allen No in irradiation you cannot say it In fact I think if one wanted to interpret our data a little more strongly than I am willing to one would have to say that there was no evidence of benefit and perhaps some possible suggestion of deleterious effect from transfusions alone

Tocantins The conditions surrounding the transfusions in your experimental animals and in man are not analogous In your animals the very fact that there were anaphylactic reactions and heparin like substances coming into the circulation makes the conditions entirely different from those in man in man one does not get those complications when well matched blood is used In the hemorrhagic disease of irradiation illness in man if one transfuses with well matched compatible blood, I dont think heparin like substances will be observed in the blood

Allen I would be willing to take the anaphylactic reactions out of it and say that there still is no evidence of benefit from blood alone in dogs

11 MILLER, C. P *et al* The treatment of postirradiation infection with antibiotics An experimental study on mice Part I and Part II AECU 1730 *(1952)*

12 DuBOIS K. P COCHRAN K. W and DOULL J Inhibition of citric acid synthesis *in vivo* by x irradiation *Proc Soc Exper Biol & Med* 76 422 *(1951)*

13 SHECHMEISTER I L BOND V P and SWIFT M N The susceptibility of irradiated mice to infection as a function of post irradiation time *J Immunol* 68 87 *(1952)*

Allen There you need hemoglobin That is a vital thing

Cronkite That is all I want you to say

Allen All right I shall say it

Cronkite That is an indication for blood There is no substitute for red cells when they are needed

Allen That is soundly established! All I am challenging is the idea that wholesale blood transfusions alone offer a real chance of reducing the hemorrhagic tendency in time of irradiation injury The blood needs must be thought through and we are sadly in need of data on this subject While transfusion seems the most reasonable and the most rational form of therapy in irradiation injury we should be able to document our ideas with data

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FIBRINOLYSIN AND ANTIFIBRINOLYSIN
A PROTEOLYTIC ENZYME SYSTEM IN BLOOD*

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Parke Davis and Company

HISTORICAL

It is nearly sixty years since Dastre (12) discovered a proteolytic principle in blood serum He observed the autolysis of whole blood clots and proposed the name fibrinolysin for the active agent responsible for the lysis Ten years later the observation was verified by Delezenne and Pozerski (34) who in their study of blood coagulation observed that chloroform increased the proteolytic activity of serum This activating effect of chloroform on serum was repeatedly verified (5678) and the conclusion was drawn that the appearance of proteolytic activity was caused by the removal of an inhibitor in the chloroform fraction which was separated after shaking the serum with this agent It is strange to say that none of these investigators reported studies on this removed fraction Yamakawa (6) even went to great lengths to extract the activators with inert solvents leaving all serum components in his activated solution

Any brief survey of the early studies on fibrinolysin in serum would not be adequate without reference to the work of Minot (9) who studied the effect of chloroform treatment upon the mechanism of blood coagulation He reported that such treatment did not affect the conversion of prothrombin to thrombin in the absence of calcium ions or added thromboplastin † Although that conclusion was challenged by Tagnon (10) and disregarded by Ferguson *et al* (11) in their earlier work we have had no difficulty in verifying the original observation in purified systems Using highly purified fibrinolysin preparations it was possible to confirm Minot's conclusion and to reaffirm the explanation of his results by Seegers and Loomis (12) It was also possible to refute Ferguson's results

* The work presented here was done in collaboration with Charles George Jr
† Author's interpretation of Minot's tables

NOMENCLATURE

I shall digress momentarily to call attention to, but not to elicit a lengthy discussion on, the naming of these compounds since it is an important problem in scientific communication. As I mentioned, Dastre (1) originally used the term fibrinolysin. Unfortunately, Tillett, Edwards and Garner (13) misnamed the compound now known to be streptokinase by first calling it streptococcal fibrinolysin. In 1945 (14) it was found that their bacterial product was really a kinase activating the proteolytic enzyme in the blood. As a result, it was promptly renamed streptokinase. Since then there has been considerable discussion, moderated by Ferguson (15), and various terms have been suggested for naming the group of compounds associated with the lytic phase of coagulation. We (16) have proposed and exclusively used the system paralleling prothrombin nomenclature. Seegers agreed to use this terminology and Lewis and Ferguson (17) have recently concluded that fibrinolysin is not a tryptase and are now using this system. Gibian (18) and others (19, 20, 21) object to fibrinolysin and insist the name should not be used because fibrin is not the exclusive substrate for the enzyme. However, we feel that fibrin is the natural physiological substrate and consequently hold to our views.

PROFIBRINOLYSIN

Recently it has been demonstrated (16, 22, 23) that this proteinase is present in plasma and serum in an inactive form, profibrinolysin, and that this precursor, having no activity of its own, can be separated from plasma or serum and then activated. Profibrinolysin has been concentrated by ammonium sulfate fractionation procedures, dialysis, isoelectric precipitation and drying (16). It may be dissolved and activated in a number of ways:

1. Profibrinolysin + $CHCl_3$ → Fibrinolysin
2. Profibrinolysin + Streptokinase → Fibrinolysin
3. Profibrinolysin + KCN → Fibrinolysin
4. Profibrinolysin + Staphylokinase → Fibrinolysin
5. Profibrinolysin + Cytofibrinolysokinase → Fibrinolysin

The best method of activation is to shake a solution of profibrinolysin with chloroform. This produces the most active bovine products. Certain strains of streptococci, notably the Lancefield type A or H46A, produce an exotoxin when grown in culture media. This exotoxin may be concentrated through certain adsorption procedures (24, 25), one of the most convenient of which is the acid

adsorption on Norite (Table XX) with elution in a 0 1 ionic strength phosphate buffer at pH 7 4 This compound after removal of the phosphate by dialysis, has the property of activating profibrinolysin and seems to be more reactive to other than bovine fibrinolysin perhaps more specifically to human material

TABLE XX

Assay of Streptokinase 307

Material assayed	Norite SG 11	Al(OH) B
	Lysis time	
Broth	8 hrs	8 hrs
Supernatant broth after adsorption	No lysis in 24 hrs	No lysis in 24 hrs
PO_4 eluate	4 hrs	16 hrs
Dialysate	230 min	16 hrs
Lyophilized (1 mg sample)	84 min	142 min

For this experiment 8 liters of sterile broth culture were divided equally and worked simultaneously Reprinted by permission from Loomis E C and Smith R W Streptococcal fibrinolysin *J Biol Chem* 163 767 (1946)

The third method of activation (KCN) is merely of academic interest as Table XXI shows the poor yield obtained in our laboratory in this manner Gerheim *et al* (26) prepared a staphylocoagulase which contained a staphylokinase and separated the two activities This staphylococcal product is most active on canine profibrinolysin Several tissue extracts have now been prepared (27 28) which by virtue of their origin and action have been given all or parts of the name represented in the fifth equation cyto fibrinolysokinase T A Loomis* (29) has produced activation by a lysokinase extracted from filter paper In our laboratory his work could not be duplicated Lundblad (30) used oxidizing agents to increase the activity of our chloroform activated bovine product

In Table XXI I should like to call attention to the fact that apparently 16 000 units of fibrinolysin were obtained from bovine serum This is much greater than any of the other preparations and points to what we now consider the species differences in fibrinolysin especially in regard to activation

* Editor's Note No kin to the author

TABLE XXI

Profibrinolysin Activation

Source	Activator	Yield	Activity U/mg N	Total Units
41 Bovine Plasma	$CHCl_3$	5 1 Gm	4 75	153
41 *Bovine Serum*	*Streptokinase*	*4 0*	*1 14*	*240*
41 Bovine Serum	KCN	4 4	1 20	528
41 Bovine Serum	$CHCl_3$	6 3	28 22	16 000

Alexander If you treat bovine plasma in which you get 153 units with chloroform and then recalcify —

Loomis These are not activations in plasma they are activations of ammonium sulfate fractionated material In other words Table XXI represents one experiment (and one duplicated many times) using four liters of serum for the last three procedures and four liters of plasma for the first In all instances the plasma and serum came from the same pooled lot

Figure 123 will show the conversion from plasma to serum but the starting material i e the material from which the plasma and serum were obtained was identical in each case

Edsall Are you going to show us what fractions were used in the ammonium sulfonate fractionated material?

Loomis Yes

Seegers Are you going to comment further on those differences of 153 and 16 000? I should like to have some idea as to why you got a total unitage of 150 in the one instance and from the serum 16 000 Do you have any idea on that at all or is that a mystery?

Loomis There is no mystery Dr Seegers In the conversion of plasma to serum we remove the fibrinogen and prothrombin by recalcifying the plasma converting prothrombin to thrombin and I trust the reaction goes to completion or to an equilibrium well along the way which removes all of the fibrinogen as fibrin As a result of recalcification the prothrombin is changed to thrombin and the latter combines with antithrombin thus making its solubility characteristics different When the ammonium sulfate fractionation is done there is less protein that precipitates Therefore a great difference in activity will be noticed in respect to nitrogen between the first and fourth product because prothrombin is gone and fibrinogen is gone which as soon as the enzyme is activated

would be digested but would still leave its derivatives to increase the nitrogen and total product weight

Seegers If I follow your reasoning then you are inclined to attach some significance to the removal of fibrinogen and perhaps to the removal of prothrombin in that the interactions after they have been completed give solubility characteristics which are quite different from those before the interactions had taken place

Loomis Right Is that sufficient?

Jensen I don t think that follows because one can test for fibrinolytic activity in plasma and serum after chloroform treatment and get more or less the same results

Seegers He was talking however of the interactions of both substances that have to do with the clotting of the blood

Alexander The conversion from plasma to serum involves alterations outside of the prothrombin conversion mechanism and deposition of a fibrin clot There are other alterations during the process of coagulation which must be borne in mind in comparing serum with the parent plasma

Seegers I think that Dr Loomis is quite right in avoiding any attempt at saying why this is so He has only said That is how I find it I was wondering whether or not there was any comment and the ones that he has made I also must admit don t satisfy my curiosity

Edsall I am still worried about this from a number of points of view For one thing I don t understand the last three items in Table XXI in each row if one compares the top and bottom rows The activity in units per milligram nitrogen in the bovine plasma is about one sixth of that in the serum The yield is comparable in the two cases Yet the total units differ by a factor of a hundred and I am not clear how that follows

Loomis This is a particular result that we obtained On the basis of this finding we have made nearly all of our fibrinolysin from serum since that time

Edsall I supposed that the activity multiplied by the yield would give the total units in some system of units but it is clear that they don t in terms of the ratios that are given there Evidently I have misunderstood something in the system of units being used

Loomis I don t know if other proteins in the plasma interfere with the activation but those are the results we obtained and we were working for the highest potency product assuming that it would be the most pure

Jensen We found that chloroform will inactivate fibrinolysin You can take fibrinolysin treat it with chloroform and destroy a considerable amount of fibrinolytic activity

Loomis After the initial activation?

Jensen Yes You start with fibrinolysin You can take human fibrinolysin obtained on activation with streptokinase treat it with chloroform and you lose quite a bit of activity

Loomis There again you are dealing with human and not with bovine

Jensen We also did it with bovine plasmin (fibrinolysin)

Loomis In chloroform activation if we went considerably beyond 48 hours we produced such loss but activation improved from 30 minutes to well beyond 48 hours possibly up to 60 hours From there on we lost activity Therefore we are more or less settled on 48 hours as the maximum activation time because with such practice we run into little difficulty in large scale preparation

Flynn Are those total units per milliliter?

Loomis Total yield

Wright Total yield from what?

Loomis The four liters of plasma or serum that were fractionated

Edsall And the total weight yield is the number of grams of protein in the fraction containing the activity?

Loomis That is right In other words 4 liters of plasma give us 5 1 Gm total weight yield That incidentally includes the salt from the physiological saline solution it was dried from

Lewis May I say one word about the differences between plasma and serum fractionation in the dog We find that when we fractionate plasma with ammonium sulfate profibrinolysin does not come out in exactly the same fraction as in serum I think this agrees with your observation that the total quantity of protein in this case the additional fibrinogen affects the action of ammonium sulfate Although the profibrinolysin lies in somewhat different ammonium sulfate fractions the total quantities in serum and plasma appear to be the same

Loomis And human comes out quite differently again I might go so far as to say that equine profibrinolysin comes out in a slightly different range all other factors being held constant

Seegers It also happens in the case of prothrombin As it becomes more and more purified the position of solubility of ammonium sulfate is tremendously altered

Edsall Yes

Seegers And that is also true for alcohol

Edsall Have you assayed for the potential activity in other fractions too in all these cases?

Loomis Yes

STABILITY OF PROFIBRINOLYSIN

It was found that several samples of profibrinolysin left standing at room temperature (26 to 28 C) gradually increased in fibrinolytic activity In the course of six months one product rose from 0 units per mg to 0 4 units per mg Samples were placed in the oven at 50 C (Figure 122) and the increase in fibrinolytic activity

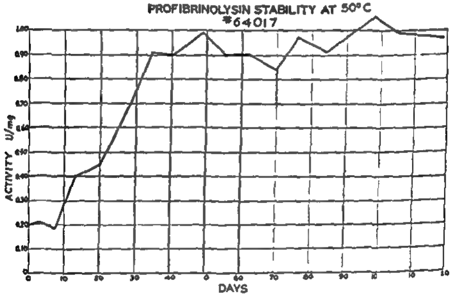

FIGURE 122

determined at intervals Since twenty one days or specifically 500 hours at 50 C is equivalent to six months at room temperature it may be seen that within one year s time a product should be fully activated and will retain that activity for a long period of time

PROPERTIES OF PROFIBRINOLYSIN

Profibrinolysin is a protein which has been prepared from the euglobulin portion of human equine bovine canine and guinea pig plasma and serum It is water insoluble saline and acid soluble and nondialyzable through a Visking or cellophane membrane On treatment with a number of agents it is converted to fibrinolysin It is relatively unstable at room temperature and much less stable at elevated temperatures Our procedure yields about 5 grams from

4 liters of plasma or serum the purity of this product being about 25 per cent as shown by electrophoretic studies (I think this will answer your previous question Dr Edsall)

PREPARATION OF FIBRINOLYSIN

Early fibrinolysin products were found to clot fibrinogen (10) Some investigators have attributed this property to the enzyme itself but it has been shown that clotting properties either direct or indirect through activation of prothrombin cannot be ascribed to fibrinolysin (12) In order to prepare prothrombin and thrombin free fibrinolysin the prothrombin and fibrinogen were removed from the plasma by adsorption on an excess of $Mg(OH)$ cream and centrifugation The procedure then as outlined in Figure 123 using this treated plasma or serum from the recalcification of oxalated plasma follows

The treated plasma or serum was cooled to 5 C a saturated solution of $(NH_4)SO_4$ added dropwise with constant stirring to 0 25 saturation The precipitated protein was removed by centrifugation for 3 minutes at 5000 r p m The solids were discarded The supernatant solution was cooled to 0 C and the degree of saturation increased to 0 30 by the further dropwise addition of saturated $(NH_4)SO_4$ with stirring The precipitate was collected by centrifugation at 5000 r p m for 3 minutes The supernatant solution was discarded

The precipitate containing the profibrinolysin was dissolved in distilled water and shaken intermittently in a separatory funnel with chloroform for 48 hours After this activation the chloroform layer was separated and discarded The aqueous phase was dialyzed for 16 hours against cold running tap water in Visking "No Jax" casings The precipitate which formed during dialysis was collected by a short fast centrifugation as above dissolved in physiological saline diluted fifteenfold with cold distilled water (to a specific resistance of about 400 ohm) cooled to 0 C and adjusted to pH 5 5 (glass electrode) with HCl The precipitate which formed was collected by centrifugation dissolved in physiological saline the solution adjusted to pH 7 0 to 7 2 with 0 1 N NaOH shell frozen and lyophilized

Are there any questions?

Alexander Yes I should like to ask again whether you obtained the very high specific activity from plasma prepared as indicated in Figure 123 and including recalcification as from plasma not recalcified but otherwise treated in an identical manner?

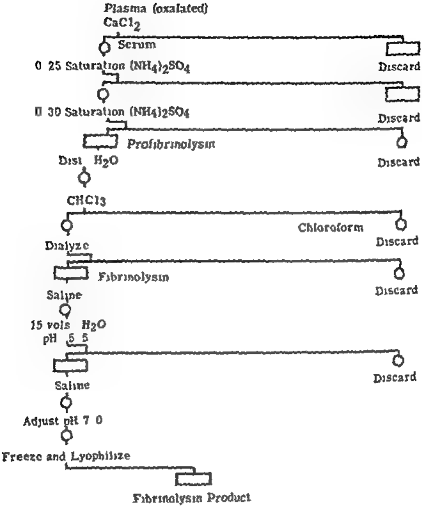

FIGURE 123 Preparation of Fibrinolysin

Loomis Yes That is exactly what we did In all of our work the blood is collected at the packing house actually large scale collection The blood is centrifuged right at the packing house and the plasma is frozen in pine kegs and shipped to us by the carload That is our starting material We take that plasma fractionate it directly or convert it to serum by recalcification or as I indicated here in some of our earlier work we removed the prothrombin and at least part of the fibrinogen by adsorption on $Mg(OH)_2$

Alexander What you call serum is really recalcified adsorbed plasma?

Loomis Not necessarily adsorbed The serum is just the product after removal of fibrin by recalcification allowing the normal clotting process to proceed in the plasma The fibrinogen precipitates as fibrin Then when the solution is separated you have a recalcified serum if you want to call it that

Alexander I have been a bit confused It was my impression that in this procedure you added calcium *after* adsorption with magnesium hydroxide

Loomis No I believe I said that we used either the treatment outlined in Figure 123 or we used magnesium hydroxide to remove prothrombin

Seegers You didn t try to add some magnesium hydroxide to the purified fibrinolysin preparation before you activated? You might have been able to adsorb quite a number of impurities

Loomis We did with no success There was no improvement in our yield in total units or in units per milligram of nitrogen We studied calcium phosphate magnesium hydroxide and silica

Seegers The interpretation would be then that you got neither an increase in specific activity nor a change in the total yield

Loomis Right One of our greatest difficulties in attempting to purify our profibrinolysin or fibrinolysin product further by this procedure is that profibrinolysin precipitates with or carries down with itself globulins of very similar characteristics I don t know how one would consider it but the proteins that are precipitated with the profibrinolysin in this fraction seem to be so nearly alike in solubility characteristics that refractionation adsorption procedures and so on do not significantly improve the purity of the product

Jensen May I answer that? Did you ever try the Harvard method of preparation? I think the human plasminogen (profibrinolysin) fraction of the Harvard group is of a higher purity than what you have here and better for some purposes

Edsall We never obtained any preparations of very high purity

Seegers Better with respect to what?

Jensen Better with respect to getting activation However we use a different method of activation We activate with trypsin because we feel that the use of chloroform does not give reliable and reproducible results One day you get 80 per cent activation the next day you get only 40 per cent since as I mentioned chloroform destroys fibrinolysin

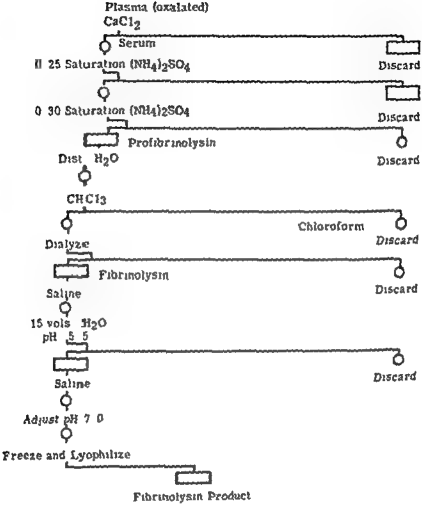

FIGURE 123 Preparation of Fibrinolysin

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PROPERTIES OF FIBRINOLYSIN

When fibrinolysin is prepared as previously described (16) by the chloroform activation of bovine profibrinolysin the yield is about 1 25 Gm per liter of plasma or serum and is about 25 per cent pure It is a nondialyzable water insoluble saline soluble euglobulin enzyme Its point of minimum solubility is pH 5 5 Arbitrarily defined one unit of fibrinolysin is that quantity which will liquefy 1 ml of a 0 3 per cent fibrin clot in 120 seconds (Figure 124) in an imidazole buffered system at pH 7 2 at 45 C A fibrin clot of this concentration was decided upon because it is comparable to a plasma or whole blood clot

ASSAY OF FIBRINOLYSIN

There appear in the literature several techniques for the assay of fibrinolysin Christensen (22) uses casein digestion with determination of the acid soluble tyrosine Schwartz and Engel (32) use the ninhydrin method and Ungar (33) has developed a very delicate spectrophotometric technique We have begun experimenting with this latter technique but have no results to present at this time Therefore we are still using the fibrinolytic assay (16) as modified (34) in the procedure for determination of antifibrinolysin This assay is based on the unit curve depicted in Figure 122

Cronkite I should like to bring up another method of assay which Dr Shulman (35) worked out while he was with Tagnon As a substrate iodinated fibrinogen is used The proteolytic activity is measured by the amount of radioactivity that is liberated into the supernatant It appears to be a very accurate measure of the degree of lysis

Loomis I don t know this method

Dr Ungar s method is intriguing and I think it will lead to much greater accuracy in fibrinolysin determinations after we get enough of the variables out of it He is working on it and getting quite excellent results We have just started

Seegers I was wondering about this setup How does that differ from what Dr Guest and I worked out?

Loomis This is essentially the same curve that you and Dr Guest worked out before you changed to 28 C

Seegers In other words it is essentially identical with our basic work

Loomis That is right

Edsall Also it was definitely a part of our findings that on the original rather crude, fraction separated by the alcohol method in which there was a good deal of lipoprotein one did get activation on treatment with chloroform However, after a later method of fractionation had first removed the lipoproteins before the fraction that contained the profibrinolysin was separated then choloroform had no effect on activation at all In this fraction there was a slow and what one may call a spontaneous activation We don t know what the activator was but it certainly was not chloroform because chloroform apparently neither accelerated nor retarded the process but did occasionally destroy some of the activity Our interpretation was that chloroform was not an activator but was probably removing an inhibitor to the activation

Loomis That is the way we would like it, too but we haven t explained it yet

Jensen We have done that experiment We used a mixture of fibrinolysin and antifibrinolysin and then treated it with chloroform and found that chloroform destroys antifibrinolysin We get fibrinolytic activity back

Loomis We have also done that In fact I reported on that in 1947 in Chicago at the Federation meeting (31)

Seegers May I ask again whether I heard that correctly that a purified fibrinolysin preparation that is to say the substance which will dissolve a clot directly when it interacts with its antisubstance from the plasma —

Jensen We used purified antiplasmin (antifibrinolysin)

Seegers All right when the combination then of the partially purified fibrinolysin and partially purified antifibrinolysin have reacted together you can then take them apart by shaking with chloroform?

Jensen Yes

Seegers That is your understanding?

Jensen Yes because the rate of destruction of the antifibrinolysin is greater than the destruction of the fibrinolysin

Loomis I am speaking from memory but I think one can get about 80 per cent recovery of lytic activity

Lewis When dog fibrinolysin and antifibrinolysin are allowed to react together we have been unable to separate them by chloroform treatment ammonium sulfate fractionation or dilution and precipitation at pH 5 5

Loomis I am talking of the whole bovine system

gradually lost its ability to clot with thrombin From these altered fibrinogen solutions the alpha and beta fibrinogen derivatives were prepared and finally active fibrinolysin recovered

Employing our fibrinolysin preparation Seegers found that fibrinolysin destroyed prothrombin (12) Figure 125 shows the rapid

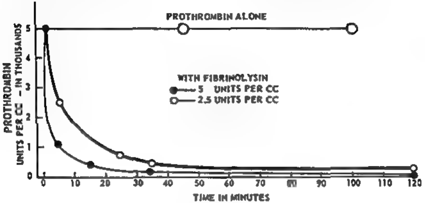

FIGURE 125 · Inactivation of prothrombin by fibrinolysin

destruction of prothrombin by two fibrinolysin solutions of different potency The prothrombin is perfectly stable but when fibrinolysin is mixed with it the activity drops off very rapidly

Prothrombin + Fibrinolysin → Inert Prothrombin + Fibrinolysin

Thrombin + Fibrinolysin → No Apparent Reaction

Prothrombin + Fibrinolysin + Serum → Active Prothrombin + Inert Fibrinolysin

The purified prothrombin is so altered by the fibrinolysin that thromboplastin and calcium ions no longer convert it to thrombin Conversely in the conversion of prothrombin to thrombin the molecule is altered in such a manner that the formed thrombin is not affected by fibrinolysin This inability of fibrinolysin to digest thrombin is an important means of differentiation in helping to classify the enzyme It was also observed that plasma and serum contained an inhibitor which prevented the destruction of prothrombin by fibrinolysin

Since fibrinolysin is an enzyme it might well be expected that there are specific and general methods for its inactivation

Fibrinolysin + Na Thioglycollate → Inert Fibrinolysin

Fibrinolysin + Guanidine HCl → Inert Fibrinolysin
or Urea

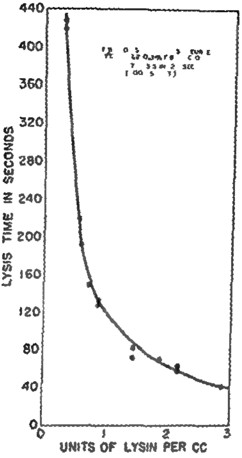

FIGURE 124

REACTIONS OF FIBRINOLYSIN

Before a fibrinolysin concentrate was available for study it was observed by the Harvard and the British Medical Research Council groups* (working on human plasma products) that alcohol fractionated fibrinogen lysed soon after clotting with thrombin We also encountered this difficulty but to a lesser degree in our early bovine fibrinogen preparations From liquefied fibrin substrates we were able to isolate the alpha and beta fibrin derivatives (36) I might say parenthetically that was Dr Seegers work while he was still at Parke, Davis

We also made our first fibrinolysin concentrates from these spent fibrin substrates We observed that fibrinogen during storage

* McFarlane A S Personal communication

Dr Loomis didn't you just finish saying that none of the reactions you listed have been reversed in your laboratory?

Loomis You caught me Yes the last one (fibrinolysin plus antifibrinolysin) has been reversed

STABILITY OF FIBRINOLYSIN

Stability studies were carried out on dry fibrinolysin products at 50 C and as shown in Figure 126 they indicate that fibrinolysin

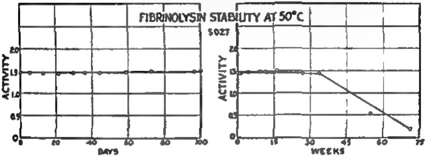

FIGURE 126

is quite stable for a considerable period of time From the graph the conclusion may be drawn that fibrinolysin is stable for twenty to twenty five weeks at 50 C or five to six years in an unaccelerated test (Except in Texas High humidity apparently is very bad for fibrinolysin and in humid areas it must be stored in a desiccator) Samples are being held for these determinations Fibrinolysin is less stable in solution The extent of such stability from our studies is 6 to 8 hours at room temperature (25 C) several days refrigerated at 3 C and several weeks at subzero temperatures (—40 C)

ANTIFIBRINOLYSIN

Hahn (38) was the first to report that normal blood serum has the power of inhibiting the action of trypsin Landsteiner (39) showed that this antienzymatic action was not precipitated with half saturation with ammonium sulfate but was present in the albumin precipitated by complete saturation with ammonium sulfate after removal of the globulin Opie and Barker (5) were the first to postulate that this antienzyme in serum held in check the activity of the serum protease currently named fibrinolysin The inhibitor compound these men worked with is presumably the compound which we called antifibrinolysin which it is a pleasure to report has been concentrated and isolated (34)

Fibrinolysin + Heat → Inert Fibrinolysin

Fibrinolysin + Plasma or Serum → Inert Fibrinolysin

Fibrinolysin + Antifibrinolysin → Inert or Neutral Protein

Reducing compounds represented in the equation by sodium thioglycollate and denaturing agents, such as guanidine hydrochloride or urea destroy fibrinolytic activity When a solution of fibrinolysin was heated to 50 C for a short interval and immediately cooled in an ice bath, its activity was destroyed Plasma serum or purified antifibrinolysin inactivate fibrinolysin Thus far, these reactions have not been reversed

Edsall What was the time and temperature in the heating process?

Loomis The time for a small volume of solution 1 ml heated for one minute at 50 C and then cooled in an ice bath

Alexander I should like to know whether sodium thioglycollate or guanidine hydrochloride or urea affect prothrombin The reason I ask is that the problem of preparing stable prothrombin preparations from human outdated plasma has bothered us a good deal and it is perfectly possible that the deterioration of prothrombin fractions may be referable to contamination with fibrinolysin It would be very helpful if we knew whether these agents could inhibit that process without affecting the prothrombin activity

Seegers I don t know what the answer is I have had no experience with guanidine hydrochloride urea, or sodium thioglycollate I expect Nanninga (37) has done some work

Your comment about human prothrombin probably not being in a suitable form for isolation in outdated plasma doesn t apply to our experience when using a method that we worked out for bovine plasma

Alexander Bovine plasma?

Seegers Outdated human plasma discarded human plasma or serologically positive plasma which nobody wants We get a specific activity for the human material which is at least equal to if not higher than that for the bovine We have had human prothrombin of a specific activity that matches anything we have ever had for the bovine material from the worst kinds of plasma source

Alexander And it is stable?

Seegers What do you mean?

Alexander Will it maintain its activity in solution for two or three weeks?

Seegers We have not done stability measurements

cold The supernatant treated serum (1 in Table XXII is cooled to 5 C and the degree of saturation increased to 0 70 by the addition of 13 5 g $(NH_4)_2SO_4$/100 ml The precipitate is collected by centrifugation in the cold dissolved in 100 ml distilled water and dialyzed 18 to 20 hours in Visking No Jax" casing 29/32 against cold running tap or deionized water

TABLE XXII

Relative Purifications of Bovine Antifibrinolysin

	Total Units	Yield
Plasma or Serum/1	60 000	100%
Supernatant Solution 1	56 000	93%
Precipitate 2	45 000	75%
Supernatant Solution 3	36 000	60%

Reprinted by permission from Loomis E C Ryder A and George C Jr Fibrinolysin and antifibrinolysin Biochemical concentration of antifibrinolysin *Arch Biochem* **20** *444 (1949)*

In Table XXII supernatant solution 1 is plasma or serum brought to 0 50 saturation with $(NH_4)_2SO_4$ dialyzed and dried from the frozen state Precipitate 2 is the 0 70 saturated $(NH_4)_2SO_4$ precipitate dissolved dialyzed and dried Supernatant solution 3 is the final dried product from the preparation

The clear dialysis residue (2 in Table XXII) is again brought to 0 50 saturation at 5 C by the addition of an equal volume of saturated $(NH_4)_2SO_4$ adjusted to pH 3 75 with N H_2SO_4 the precipitate removed by centrifugation in the cold and the supernatant solution neutralized to pH 7 0 with N NaOH The neutral solution is again dialyzed as above and dried from the frozen state (3 in Table XXII)

Seegers You mean the precipitate?

Loomis No nothing comes out of solution on dialysis A clear solution results

Edsall You are just getting rid of the salt at that stage?

Loomis We are just getting rid of it We take the entire solution and bring it back up to 50 per cent of saturation with ammonium sulfate

Seegers May I ask why you want to get rid of the salt? Didn t you just have ammonium sulfate fractionation?

PREPARATION OF ANTIFIBRINOLYSIN

Antifibrinolysin has been prepared from bovine equine and human plasmas as well as from their serums by the method outlined in Figure 127. Slightly better results have been obtained from

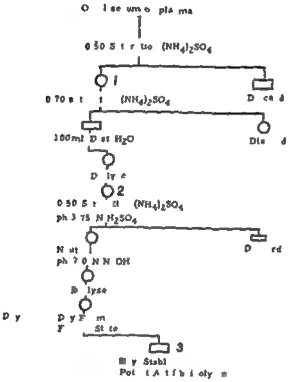

FIGURE 127 Preparation of Antifibrinolysin

oxalated rather than citrated plasmas but it is not considered to be a critical difference. When serums were used they were prepared from oxalated plasmas as previously described (13). Temperature control below 26° C is not an absolute necessity but it is felt that solubility factors are reduced with lower temperatures. Hydrogen ion adjustment on the acidic precipitation is critical and affords only a narrow range between pH 3.5 and 4.0.

One liter of bovine serum is cooled to 5° C and brought to 0.50 saturation by the addition of an equal volume of saturated $(NH_4)_2SO_4$. The precipitate is removed by centrifugation in the

Jensen We have two and the antifibrinolytic activity was found to be present in the fast moving component No antifibrinolytic activity was ever observed in the slower component

Loomis I am speaking from memory but it seems to me there is one fast moving component that clears the field very soon Then one has the major peak and one other besides the starting boundary

Jensen We feel that our antifibrinolysin preparation still contains albumin

Loomis Possibly so

Edsall What is the mobility of the main peak?

Loomis We have those data but I do not have them here

Edsall I cannot help wondering very seriously about the question of purity in situations of this sort If the active component was only a very small fraction of one per cent of the main peak it might still be all in that main peak Yet the actual purification might be at a very early stage indeed I think

Jensen I agree with Dr Edsall We have compared our antifibrinolysin preparation with crystalline trypsin inhibitor from soy beans and on the basis of this comparison our antifibrinolysin is still rather impure

Loomis We have since prepared electrophoretically homogeneous material whose specific activity that is units per milligram of product without any salt runs in the neighborhood of 25 to 30 units per milligram That is the purest we have been able to get it Electrophoretically it is homogeneous I would assume its purity to be at least 90 per cent or better but I hesitate to say that it is pure material

Seegers There is always a good way to get around that one and that is that the fellow who claims that there might be 1 per cent should be asked to go ahead and get the rest out That is the only defense one has against that kind of question

Jensen That is exactly what we are doing

Edsall There are other tests of purity for example the solubility test homogeneity in the ultracentrifuge as well as by electrophoresis

Seegers You can do all those and still get the fast curve It is best to throw it right back

Jaques May I ask about the comparison of antifibrinolysin and crystalline trypsin inhibitor? Was that on the basis of antifibrinolytic activity?

Loomis Yes

Jaques Not against trypsin?

Loomis In the precipitation preceding dialysis the viscosity of the 0 70 saturated ammonium sulfate solution is very high and the precipitate does not pack tightly Therefore the precipitate carries with it a variable though *sizable quantity of ammonium sulfate* These observations caused us to include the dialysis step to remove the salt before advancing to the next fractionation

Edsall That is essentially in order to know what the concentration of ammonium sulfate is in the next step

Loomis That is right We take the saturation up to 50 per cent and adjust the pH to 3 75 with normal sulfuric acid remove the precipitate by centrifugation in the cold and neutralize the supernatant solution to pH 7 with *sodium hydroxide* The neutral solution is then dialyzed as above and dried from the frozen state

Jensen We have done this too according to your directions but we find that the preparation we get still contains quite a bit of antithrombin activity The antifibrinolysin preparation you kindly sent us did not We have carried the separation a little bit further We have dissolved the above preparation in 0 5 saturated ammonium sulfate brought the supernatant to 0 6 ammonium sulfate saturation and the supernatant of the 0 5 to 0 6 precipitate to 0 7 saturation In the 0 5 to 0 6 fraction we find mainly the antithrombic activity and in the 0 6 to 0 7 fraction the antifibrinolytic activity By repeating this procedure we get a fairly purified antifibrinolysin preparation

Loomis By just repeating ammonium sulfate fractionation?

Jensen We actually go through the whole process We dissolve in 0 5 ammonium sulfate adjust the pH to 3 2 and after removing any precipitate readjust the pH to 5 5 and bring the solution to 0 6 and the supernatant of the 0 6 precipitate to 0 7 ammonium sulfate saturation We found that the 0 5 to 0 6 fraction was quite high in antithrombic activity

Loomis We have never had antithrombin in our material

Jensen The material you sent us had no antithrombic activity

Loomis Table XXII shows the relative purification at the steps indicated in the process flow sheet Figure 127 This material is about 80 per cent pure by electrophoresis

Edsall There is 80 per cent of one component?

Loomis Right

Edsall And that component is the one that contains the activity?

Loomis That is the activity as far as we know

Jensen You have only one component in the electrophoresis?

Loomis No I believe there are three in the final product

Jensen We have two and the antifibrinolytic activity was found to be present in the fast moving component No antifibrinolytic activity was ever observed in the slower component

Loomis I am speaking from memory but it seems to me there is one fast moving component that clears the field very soon Then one has the major peak and one other besides the starting boundary

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Seegers You can do all those and still get the fast curve It is best to throw it right back

Jaques May I ask about the comparison of antifibrinolysin and crystalline trypsin inhibitor? Was that on the basis of antifibrinolytic activity?

Loomis Yes

Jaques Not against trypsin?

Loomis No that is antifibrinolysin

Kunitz's (40) preparation of soybean trypsin inhibitor in our fibrinolysin and antifibrinolysin assay runs about 225 units per milligram As I just said the best antifibrinolysin preparations that we have ever made and which we suspect are essentially pure run about 30

PROPERTIES OF ANTIFIBRINOLYSIN

Antifibrinolysin has been prepared from human equine and bovine plasmas and their serums It is a water soluble nondialyzable protein antienzyme It is not an antithrombin It is stable for two years in the dry state at 26 C and several months at subzero temperatures The average yield from 1 liter of serum or plasma is 10 Gm of crude preparation about 20 per cent pure 1 Gm of a product 60 to 70 per cent pure and 0 1 Gm of pure antifibrinolysin with reservations on that pure

Antifibrinolysin has been found to be a potent antienzyme blocking the destruction of fibrin fibrinogen and prothrombin by fibrinolysin

TABLE XXIII

Destruction of Fibrinolysin by Antifibrinolysin

Antifibrinolysin Preparation	Units Fibrinolysin Added	Units Fibrinolysin Remaining 30 min	60 min	90 min
422	7 13	2 20	0 44	0 42
403	8 30	7 50	7 50	7 39
403	7 33	3 73	3 20	3 17
409	7 78	3 73	3 30	3 28
410	10 80	5 00	3 30	3 22
412	8 04	7 50	6 50	6 48
415	9 47	1 45	0 42	0 38

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The inactivation of fibrinolysin by antifibrinolysin (Table XXIII) takes place rapidly *in vivo* and the neutralization *in vitro* may be considered complete in 60 minutes This is undoubtedly slower than in vivo since no trace of active injected fibrinolysin has been recovered From the data it is apparent that the neutralization is

not quite complete in 30 minutes Consequently we have defined one unit of antifibrinolysin as that quantity which will neutralize one unit of fibrinolysin in 60 minutes in a saline solution buffered at pH 7 2 with imidazole the reaction proceeding at room temperature In practice an excess of fibrinolysin is added to the antifibrinolysin preparation and the amount of antifibrinolysin assayed by loss of activity of the fibrinolysin

STABILITY OF ANTIFIBRINOLYSIN

Stability studies on various of our products heated in the oven at 50 C show that antifibrinolysin is a stable preparation There can be no question concerning its stability at 50 C for at least 40 days This would indicate a stability of two years at room temperature Figure 128 presents this data

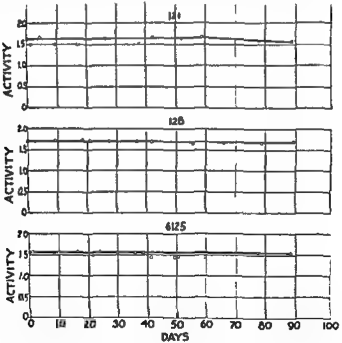

FIGURE 128 Antifibrinolysin stability at 50 C for three preparations

RELATION OF THESE COMPOUNDS TO THE CLOTTING MECHANISM

From the presentation thus far it becomes apparent that fibrinolysin and antifibrinolysin are interrelated in the clotting mechanism

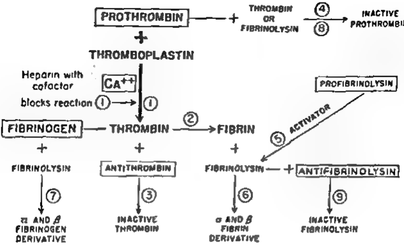

Figure 129 Simplified clotting diagram

Figure 129 shows the known reactions If we start with reaction 5 in this Figure the activation of profibrinolysin to fibrinolysin by such activators as chloroform streptokinase staphylokinase or cytofibrinolysokinases we have the enzyme to proceed to reaction 6 the destruction of a fibrin clot by fibrinolysin Reaction 7 shows the parallel effect on fibrinogen In both cases the end products are the alpha and beta derivatives from the substrate proteins Reaction 8 calls attention to the inactivation of prothrombin by fibrinolysin and reaction 9 indicates the formation of a protein complex of fibrinolysin and antifibrinolysin which possesses neither activity if reacted on a unit basis Work is advancing with this protein but it has not yet been isolated or crystallized

Wright Dr Lewis will open this discussion

Lewis We have been studying the fibrinolytic enzyme system for about four years with the main purpose of determining two things why this system exists and how it functions * My first interest in the fibrinolytic enzyme system was aroused by an observation of whole blood clot lysis in a case of phenobarbital poisoning Since that time in a very limited clinical series I have seen this phenomenon of whole blood clot lysis premortem in 5 cases of severe methyl alcohol poisoning (4 of which eventually proved fatal) 3 cases of severe shock 1 coronary occlusion and 4 severe cirrhotics As our clinical facilities are rather limited we decided to study the

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health U S Public Health Service

dog Our study has been divided into two phases first *in vivo* studies reproducing some of these clinical syndromes and second *in vitro* studies of the component parts of this enzyme system

Our *in vivo* studies have been quite disappointing Our first series was that of a group of dogs subjected to hemorrhagic shock Sixteen dogs received large arterial bleedings followed by repeated bleedings or slight infusions of saline or blood whichever was needed to maintain the blood pressure at the so called shock levels The dogs survived anywhere from 22 minutes to 5 hours In this group of 16 dogs we observed 5 that showed whole blood clot lysis

Edsall What do you mean by whole blood clot lysis? Could you define it?

Lewis Yes whole blood clot lysis is defined as the complete disappearance of a clot in 2 ml of blood incubated at 37 C in a period of 24 hours or less Specimens with definite gross contamination are discarded We see whole blood clot lysis extremely rarely

In this first group of 16 dogs antifibrinolysin and profibrinolysin titers were followed in only 2 dogs One showed no significant changes whereas the other dog showed a marked drop in antilysin immediately before death and in the specimen of the heart s blood This heart s blood did not clot at all and contained no fibrinogen The prolysin contents of both of these samples were markedly reduced

The next experiment consisted of 30 dogs which were bled rapidly to death the blood being employed in other experiments In these dogs we simply took a final sample from the heart s blood and incubated it for 24 hours No whole blood clot lysis was observed in any of these 30 dogs This series has now been extended to include a great many more dogs and whole blood clot lysis has not been seen in any sample

In our third group of "hemorrhagic shock" dogs we attempted to follow a definite plan and to study these dogs in some detail We took 10 dogs bled them initially 3 per cent of their body weight followed 2 hours later by a second bleeding of 1 to 2 per cent of their body weight Blood samples were obtained at hourly intervals thereafter These dogs survived the second bleeding anywhere from 5? minutes to 3½ hours The results were disappointing in that we found no whole blood clot lysis and no significant changes in fibrinogen antifibrinolysin profibrinolysin or serum fibrinolysokinase concentrations

Our next experiment was an attempt to produce methyl alcohol poisoning Four dogs were given 7 to 9 ml of methyl alcohol per

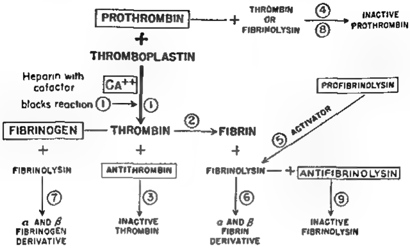

FIGURE 129 Simplified clotting diagram

Figure 129 shows the known reactions If we start with reaction 5 in this Figure the activation of profibrinolysin to fibrinolysin by such activators as chloroform streptokinase staphylokinase or cytofibrinolysokinases we have the enzyme to proceed to reaction 6 the destruction of a fibrin clot by fibrinolysin Reaction 7 shows the parallel effect on fibrinogen In both cases the end products are the alpha and beta derivatives from the substrate proteins Reaction 8 calls attention to the inactivation of prothrombin by fibrinolysin and reaction 9 indicates the formation of a protein complex of fibrinolysin and antifibrinolysin which possesses neither activity if reacted on a unit basis Work is advancing with this protein but it has not yet been isolated or crystallized

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the other thing is some recent work that Dr Landy in our laboratory has done injecting thromboplastin intravenously in dogs They go through a very extensive lytic phenomenon in about 2 and 4 hours which then tends to disappear (43)

Alexander May I add for the record some observations made in Europe Dr Soulier and Dr Le Bolloch who is at present working in my laboratory have reported a considerable series of patients who after being subjected to pulmonary surgery developed marked fibrinolytic activity with hemorrhagic sequelae (44) I do not know of observations of that sort in the United States

Seegers Was it also Soulier's laboratory that made the observation that acetylcholine induced shock would produce lysin?

Jensen That is correct Soulier and Koupernik (45) reported constant occurrence of fibrinolysis during acetylcholine induced shock

Alexander Theirs is a considerable series of cases and I mention it because I think we in this country ought to be alerted to the disorder as a complication of pulmonary surgery

Tocantins Patients undergoing pulmonary surgery receive large amounts of blood don't they?

Alexander The complication occurs in the absence of shock

Allen In the past the Europeans have not given much blood for shock

Cronkite Apparently they do from the blood bank service in Paris

Alexander Also it is not in relation to transfusion They have observed it in pulmonary manipulation or pneumolysis in which there is no question of hemorrhage or of treatment with blood replacement therapy

Tocantins We have observed a similar phenomenon when we inject intravenously euglobulin solutions prepared by the dilution and acidification method within an hour or two even when the clotting time is accelerated by the injection the clots liquefy very rapidly

Lewis I think if I may go on that our *in vitro* studies may suggest answers to some of these problems

Figure 130 shows the fibrinolytic enzyme system of dog serum Our *in vitro* studies have suggested that the fibrinolytic enzyme system consists of at least five components *fibrinolysin* which is not present in normal serum *profibrinolysin* the precursor which may be activated to the fibrinolysin by *fibrinolysokinase* and two inhibitors *antifibrinolysin* which inactivates fibrinolysin and *anti*

kilogram subcutaneously Two of these dogs died 52 and 76 hours after injection in coma with a shocklike picture Two survived the coma period but died subsequently with infection from large ulcers at the sites of injections No whole blood lysis and no significant changes in the antilysin titer were found

In one dog we tried to reproduce some of MacFarlane's (41) observations by injecting adrenalin but could detect no changes in the fibrinolytic enzyme system

In the last group of dogs we tried to produce severe liver damage by injecting carbon tetrachloride into the portal system Ten dogs received 0 15 ml per Kg of carbon tetrachloride undiluted into the portal system All of these dogs developed evidence of severe liver damage shown by marked changes in BSP and the Quick one stage prothrombin test In one dog approximately 3 days after injection no fibrinogen could be found in the blood and there was a very low antilysin titer although the serum itself showed no active fibrinolysin when tested in a standard fibrin clot (42) We have not completed the studies on these dogs and the specimens have been frozen for more extensive studies of the clotting and fibrinolytic systems

Flynn Have you tried intracranial injuries? In the Army during World War II some of the head injury cases we autopsied had incoagulable blood suggesting the presence of a fibrinolysin

Lewis We hope to be able to study human cases soon Apparently humans exhibit fibrinolytic phenomena more readily than dogs

Seegers May I ask once more how you produced the liver damage with carbon tetrachloride

Lewis We put it into a mesenteric vessel at operation

Allen Were the shock patients you spoke of crush injuries extensive surgery or what?

Lewis Two of them were vaginal hysterectomies with very extensive hemorrhage Venous blood samples clotted and then the clot disappeared in anywhere from one to six hours In one patient no clotting at all was observed in several samples suggesting that the fibrinogen may have been destroyed *in vivo* Unfortunately more extensive tests were not made and the patients were not studied for anticoagulants which in view of Dr Allen's work might also have been present At least one blood sample from each of these patients was seen to clot and lyse

Allen There are two reasons I asked my last question One is that we have observed and reported on 18 cases in humans and

to two weeks The fibrinolytic activity is tested repeatedly and when it reaches a plateau the fraction is tested for residual pro fibrinolysin by addition of staphylokinase

I should like to emphasize three things concerning Figure 131 Dilutions of fibrinolysin lyse the standard amount of fibrinogen (100 units) in times which follow closely a simple inverse relation ship between the concentration of fibrinolysin and the lysis time We have defined 100 units of fibrinolysin as that amount which will dissolve 100 units of fibrinogen (approximately 3 mg) in exactly 5 minutes at 37 C and pH 7 7 The relationship between fibrinolysin concentration and lysis time may be expressed by the formula $\text{units of fibrinolysin} = \frac{500}{\text{lysis time}}$ The experimental points follow the theoretical curve quite closely

The second point is that the lysis time is independent of the con centration of thrombin employed in the test

The third point deals with the relationship between fibrinogen concentration and lysis time Over a rather narrow range lysis time appears to increase in direct proportion to increases in fibrinogen concentration (42 46) $\left(\text{fibrinogen units} = \frac{\text{lysis time} \times 100}{5}\right)$ Actually if fibrinogen concentration is increased beyond this narrow range the direct relationship no longer exists and the experimental points form a curve This might be explained if we assume that fibrinolysin and fibrinogen form an intermediary compound before lysis of fibrinogen (or fibrin) occurs

Seegers There is something I want to ask you We had this type of curve extrapolated to another unit system and had also worked out the concentration of fibrinogen which I think is almost identical to your results What is it that prompts people to re-do these things in a whole new set of systems?

Lewis That is very simple Dr Seegers We had been working on this system for a couple of years at the time your paper was published We had our own data Why should we change to an already published method?

Seegers I wasn t aware that you had been working on this I thought you were in some place other than Chapel Hill at that time I ask because it is the same thing in the case of thrombin Everyone has his own thrombin unit

Lewis Before going to Chapel Hill I was using the first widely accepted method that of Kaplan (47) We simply modified his

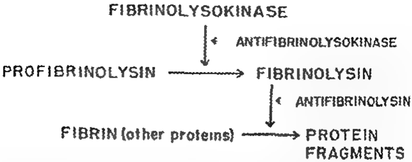

FIGURE 130 Fibrinolytic enzyme system of dog serum Reprinted by permission from Lewis J H and Ferguson J H Studies on a proteolytic enzyme system of the blood IV Activation of profibrinolysin by serum fibrinolysokinase *Proc Soc Exper Biol & Med 78 184 (1951)*

fibrinolysokinase which prevents the conversion of profibrinolysin to fibrinolysin This inhibitor has not yet been isolated and studied but we have some indirect evidence that one (or more) may exist

We prepare fibrinolysin by obtaining the euglobulin fraction of dog serum diluted and precipitated at pH 5 5 After reprecipitation this fraction contains no detectable antifibrinolysin and little fibrinolytic activity It is allowed to activate spontaneously at 5 C for one

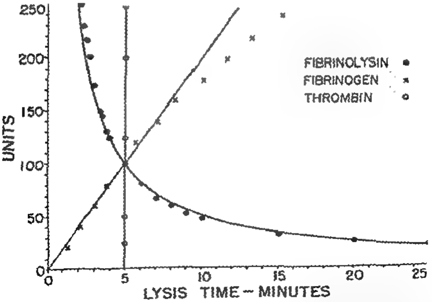

FIGURE 131 Relationships between concentrations of fibrinolysin fibrinogen and thrombin Points are experimental. Smooth curves are the calculated theoretical curves

TABLE XXX

Fibrinogenolytic Action of Trypsin

Enzyme Units per Test	Fibrinolytic Time	Fibrinogenolytic† Time
Fibrinolysin 93 units	5 10	5 15"
Trypsin 104 units (75 μg)	4 45	15
62.4 units (45 μg)	[illegible] 40	30
41 6 units (30 μg)	18 30	40
20 8 units (15 μg)	over 2 hours	2 30

Lysis time of standard clot

† Time at which no clot is observed after adding aliquot (0 4 ml.) of fibrinogen enzyme-buffer mixture to [illegible]00 unit thrombin (0 1 ml.) Concentrations of fibrinogen and enzyme are the same for fibrinolytic and fibrinogenolytic tests

required to dissolve the fibrin whereas an equal quantity of fibrinogen was destroyed (i e would not clot on addition of strong thrombin) in between 10 and 15 seconds After the trypsin had been diluted to 15 μg per test the fibrinolysis time was over 2 hours but the fibrinogenolysis time was only 2½ minutes

Alexander Is the trypsin devoid of metal?

Lewis No it is not

Alexander So that it is really not strictly comparable until magnesium has been added to the fibrinolysin

Lewis That is true We were interested here in comparing fibrinolytic and fibrinogenolytic times Fibrinolysin destroys fibrin and fibrinogen at essentially the same rate but trypsin destroys fibrinogen much more rapidly than fibrin

Alexander I was curious to know whether the cation in the crystalline trypsin might not in part explain some of the differences between the action of trypsin and fibrinolysin in that regard

Lewis It possibly could We felt that we were using this trypsin in such a dilute solution that the effects of the small amount of magnesium remaining would not be significant Addition of magnesium to fibrinolysin does not alter the fibrinolysis time We have not tested its effects on fibrinogenolysis

Jaques What preparation of trypsin?

Lewis It was a preparation obtained from M Kunitz of the Rockefeller Institute

method and were using it at the time that you published your first paper

Seegers In the case of thrombin there is not a single investigator who has used the same thrombin unit It is always different

Edsall We tried to use the same Iowa unit that you did

Seegers Much could be said about that

Edsall Yes

Seegers Apparently, the same thing is going to happen with fibrinolysin You say now, in essence that it was a matter of convenience

Lewis It is very hard to take two years of work and tear it up because someone else has published it You just don t do that

Fremont Smith There is always something nice and familiar about one s own

Seegers There certainly is

Lewis We were planning to study the effects of certain inhibitors on fibrinolysin and thought it would also be interesting to study the effects on trypsin a well known proteolytic enzyme Our first attempts to study trypsin in this system gave erratic results due to two things first that trypsin is thrombinolytic and second that trypsin destroys fibrinogen much more rapidly than fibrin

TABLE XXIV

Thrombinolytic Action of Trypsin

Test Mixture	INCUBATION PERIOD				
125 units Thrombin +	15 sec	3 min	30 min	60 min	24 hr
Buffer	4 5	4 4	4 3	5 1	6 6
125 units Fibrinolysin	4 4	4 3	4 3	4 5	7 3
121 units Trypsin	4 4	6 4	17 5	30 3	480 0

Clotting times (seconds) after addition of 0 25 ml test mixture to 0 5 ml fibrinogen Test mixture incubated at 29 C for stated intervals

Table XXIV shows rather simply the thrombinolytic action of trypsin The observation that fibrinolysin has a negligible thrombinolytic effect is I believe in agreement with Dr Loomis

We were quite surprised to find that trypsin destroys fibrinogen much more rapidly than fibrin Table XXV shows that fibrinolysin (98 units per test) destroys fibrin and fibrinogen at approximately the same rate In the first trypsin test (104 units), 4 minutes were

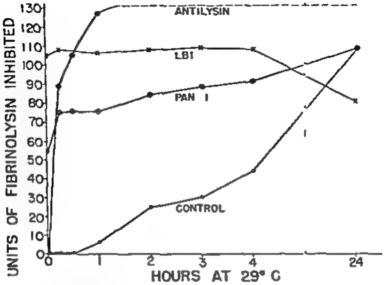

FIGURE 133 Inhibition of fibrinolysin at 29 C by antilysin (concentration approximately 1/12 normal serum) lima bean inhibitor LBI (0 007%) and pancreatic inhibitor Pan I (0 04%)

units of fibrinolysin destroyed is plotted against the time of incubation First note that our control fibrinolysin is not stable over the 24 hour incubation period it has lost about 75 per cent of its activity It takes about 48 hours to lose complete activity Second note that antilysin in the strength used here which is approximately one twelfth that of whole serum produces no immediate inhibitory action but that the inhibitory action rapidly increases until the fibrinolysin is well over 80 per cent inhibited We cannot measure very accurately the difference between 90 per cent and 100 per cent inhibition

Lima bean inhibitor exerted its full inhibitory action immediately Another point to note about lima bean inhibitor is that at the 24 hour period there was apparently a release of fibrinolysin and this test mixture actually has more fibrinolytic activity than the control.

The pancreatic inhibitor also showed almost full inhibitory effect immediately and only a slight increase was noted thereafter

We were rather intrigued by the increase in fibrinolysin titer after 24 hour incubation with lima bean inhibitor and theorized that it might be due to an inhibition of antilysin present as a contaminant in our fibrinolysin although we had been very careful in our prepa

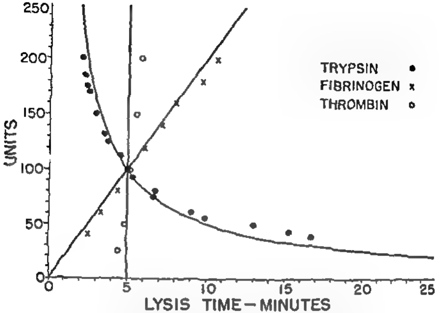

FIGURE 132 Relationships between concentrations of trypsin fibrinogen and thrombin Points are experimental smooth curves are the calculated theoretical curves

Jaques A crystalline preparation?

Lewis Yes

Figure 132 shows the relationships between trypsin fibrinogen and thrombin in our fibrinolytic system In view of the marked fibrinogenolytic and thrombinolytic effects of trypsin our standard fibrinolytic technique was slightly modified A drop (0 04 ml) of 1000 unit thrombin was placed on the side of the tube containing the buffered fibrinogen The fibrinogen was then poured over the thrombin and into a tube containing the trypsin and the mixture immediately poured back Thus mixing and clotting took place almost simultaneously and by standardization of the procedure we attempted to keep the error due to fibrinogenolysis constant and minimal The curves show that trypsin reacts similarly to fibrinolysin in this fibrinolytic test

We were interested in studying the inhibition of fibrinolysin and trypsin by three different types of inhibitors (*a*) the serum inhibitor either as whole serum or as a purified fraction called antilysin (*b*) a vegetable inhibitor lima bean inhibitor which is similar to soybean and navy bean inhibitors but is heat stable and (*c*) the well known pancreatic trypsin inhibitor

Figure 133 shows the effect of these inhibitors when mixed with equal amounts of fibrinolysin and incubated at 29° C The number of

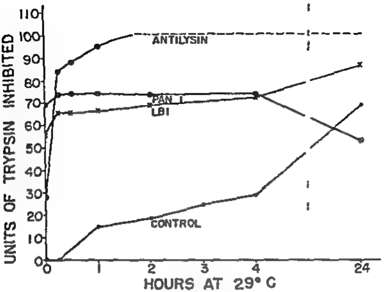

FIGURE 135 Inhibition of trypsin by antilysin (1/3.5 normal serum concentration) LBI (0.00.5%) and pancreatic inhibitor (0.0024%)

of fibrinolysin and show that trypsin is more readily inhibited by serum antifibrinolysin and by pancreatic inhibitor The shapes of the curves are similar to fibrinolysin Antilysin does produce some initial inhibiton followed by a rapid loss of trypsin activity Lima bean and pancreatic inhibitors produce most of their inhibitory effects immediately

Figure 136 shows the effect of increasing amounts of lima bean inhibitor when added to trypsin The amounts of trypsin inhibited are directly proportional to the amounts of lima bean inhibitor added

In contrast to this when increasing amounts of lima bean inhibitor are added to fibrinolysin a direct proportionality is not obtained The experimental points (Figure 137) follow quite closely a theoretical curve which was calculated for a reversible reaction obeying the law of mass action between lima bean inhibitor and fibrinolysin The inhibition of fibrinolysin by lima bean inhibitor closely resembles the inhibition of chymotrypsin by soybean inhibitor (48)

Increasing amounts of pancreatic inhibitor (Figure 138) inhibit trypsin in direct proportion to the amount of pancreatic inhibitor present Note the low concentrations of pancreatic inhibitor necessary to inhibit trypsin

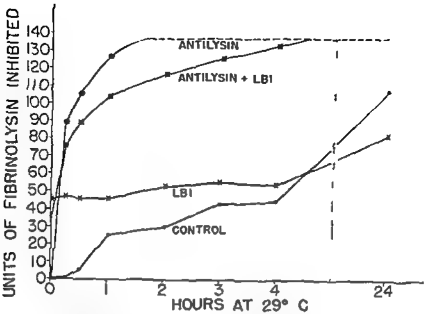

FIGURE 134 Inhibition of fibrinolysin by antilysin (1/12 serum concentration) lima bean inhibitor (0 0015%) and a mixture of antilysin (1/12 serum concentration) and LBI (0 0015%)

ration of the fibrinolysin and tests made while it was still in the inactive stage had shown no detectable antifibrinolysin

The experiment of Figure 134 was undertaken to see whether lima bean inhibitor would actually inhibit antifibrinolysin The control fibrinolysin slowly deteriorated showing marked loss of activity at 24 hours The mixture of lima bean inhibitor and fibrinolysin showed an immediate decrease in activity but at 24 hours there was more activity than in the control Antilysin mixed with this fibrinolysin again produced a rapid disappearance of fibrinolytic activity If a mixture of the same amounts of antilysin and lima bean inhibitor was added to fibrinolysin the inhibiting action of antilysin was decreased It did not seem to make any difference in our experiments whether we mixed the antilysin and lima bean inhibitor first or simultaneously with the addition to fibrinolysin

Loomis Dr Lewis is the dotted line on the antilysin curves on these Figures an extrapolation?

Lewis No We are unable to measure 0 to 10 units of fibrinolysin accurately The dotted line indicates that fibrinolytic activity is less than 10 units per ml

Figure 135 shows the inhibition of trypsin by the three inhibitors The concentrations are different from those used in the inhibition

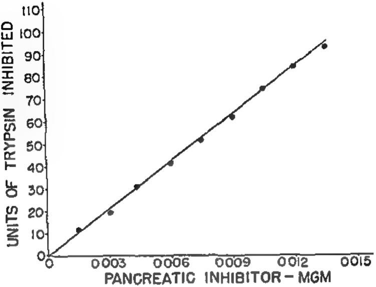

FIGURE 138 Inhibition of trypsin by various concentrations of pancreatic inhibitor

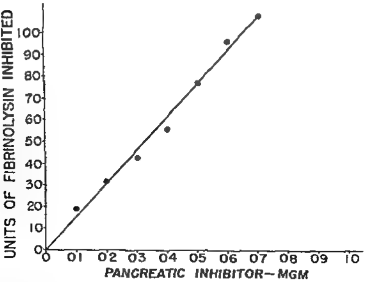

FIGURE 139 Inhibition of fibrinolysin by various concentrations of pancreatic inhibitor

FIGURE 136 Inhibition of trypsin by various concentrations of lima bean inhibitor

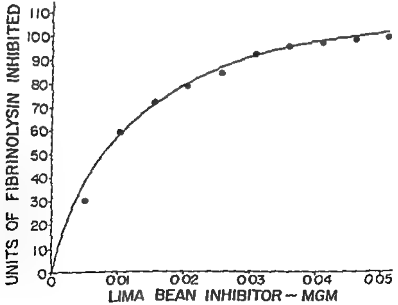

FIGURE 137 Inhibition of fibrinolysin by various concentrations of lima bean inhibitor Points experimental smooth curve theoretical

apparently has no fibrinolysokinase activity even for its homologous profibrinolysin

FIBRINOLYSOKINASE

BACTERIAL
Streptokinase — Acts only on human
Staphylokinase — Acts on human dog cat rabbit guinea pig
Does not act on bovine or rat
TISSUE
Lung (from human dog cat rabbit guinea pig rat mouse
not from bovine) — Acts on all
Uterus ureter bladder gall bladder lymph gland ovary (from dog)
SERUM

Seegers Did you get the material as an extract from these tissues or did you use the whole tissue?

Lewis We used differential centrifugation methods modified from Claude (51 52) and prepared 8 fractions from each tissue The extract that is the final supernatant showed no activity and frequently some inhibitor activity Most of the kinase activity was concentrated in the fractions which we have called 4 and 6 and which are approximately equivalent to the large and small granule fractions of Claude the mitochondrial and microsome fractions

In special studies (53) of dog lung we attempted to purify the kinase and study its properties Fresh lung was perfused macerated and ground The mixture was centrifuged at 4000 g for 15 minutes and the sediment discarded The supernatant was then centrifuged at 40 000 g and the sediment collected and washed by suspension in a buffered saline (pH 7 3) solution and recentrifugation This sediment consisting of large and small cell granules was dialyzed against 0 0001 NaOH for 24 hours in the cold The dialyzed material was again centrifuged at 40 000 g and the sediment discarded The supernatant was brought to pH 3 8 and the flocculant precipitate collected Reprecipitation was carried out one or more times This material which appeared to be a protein with isoelectric point at approximately pH 3 8 was used in most of our experiments

Jensen How long do you incubate your profibrinolysin with this tissue kinase?

Lewis That will be answered shortly

We have found it more advantageous to incubate activation mixtures for long periods of time at 4 to 6 C as this prevents to a large extent the deterioration of fibrinolysin The next three Figures demonstrate experiments on the activation of profibrinolysin by staphylokinase Figure 140 shows that at all the pH levels studied

Figure 139 shows that fibrinolysin is inhibited by pancreatic trypsin inhibitor in direct proportion to the amount of inhibitor present Note that *approximately one hundred times as much* pancreatic inhibitor is necessary to inhibit fibrinolysin as is necessary to produce the same effect on trypsin

I do not have figures to illustrate the reaction between antifibrinolysin and trypsin or fibrinolysin Apparently very strong antilysin preparations either as whole serum or as the isolated antilysin fraction will completely inhibit up to 200 units of fibrinolysin immediately As we use weaker and weaker concentrations of inhibitor the point of complete inhibition is reached at various times which seems to be dependent upon the concentration of antilysin present Although these are still preliminary experiments *they differ somewhat from our own previous observations* (42) and those of other workers (46 49) and suggest that the action of antilysin on fibrinolysin is perhaps similar to that of an enzyme that is the rate of the reaction but not the final amount of fibrinolysin inhibited is dependent upon the concentration of antifibrinolysin

In studying the inhibition of trypsin by whole serum and by antilysin we obtained quite divergent results Antilysin the purified fraction inhibited trypsin much the same as it does fibrinolysin but whole serum did not The reaction with whole serum suggested *that trypsin actually was activating the profibrinolysin present* in the serum and separate tests have shown that trypsin does activate dog profibrinolysin

Next I should like to discuss the activation of profibrinolysin In many experiments we have been unable to obtain any evidence that profibrinolysin activates spontaneously (i e autocatalytically) A fibrinolysokinase appears to be necessary We have classified the known fibrinolysokinases as bacterial tissue and *serum Trypsin* should be added to this list Note that we do not include chloro*form* It does not activate the isolated serum kinase free profibrinolysin (dog) Our preparations of streptokinase have activated only human profibrinolysin whereas staphylokinase (50) activates human dog cat rabbit and guinea pig prolysin but not bovine or rat We studied thirty three different types of dog tissue (27) preparing eight fractions from each tissue *High fibrinolysokinase* activity was found in the tissues listed below Lung tissue proved a particularly convenient *source as it may readily* be freed from blood by perfusion with saline and contains high kinase activity We have studied the lung from a number of different species and find that all are active with the exception of bovine lung which

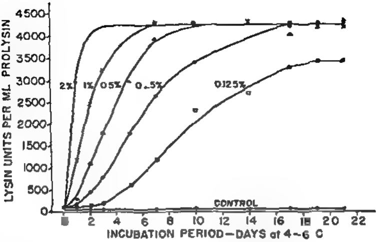

FIGURE 141 Activation of profibrinolysin by staphylokinase Effect of varying staphylokinase concentration Reprinted by permission from Lewis J H and Ferguson J H A proteolytic enzyme system of the blood III Activation of dog serum profibrinolysin in staphylokinase *Am J Physiol* 166 594 (1951)

Lewis Well staphylokinase (2 per cent) kept at this temperature is fairly stable but as it is diluted down to the levels that you speak of (0 125 per cent) it does lose some activity That is why we haven t put a great deal of weight upon the fact that weaker concentrations usually do not reach the maximal level of fibrinolysin yield We are not sure that the staphylokinase itself has not deteriorated

Edsall Is there any indication of inactivation by metallic ions for instance?

Lewis No We have tried a number of experiments to see whether metallic ions either aided or hindered activation by staphylokinase and also by tissue kinase We haven t tried it on the serum kinase As far as we can tell activation of profibrinolysin is not affected Many ions particularly in high concentration affect our test system that is the formation of the fibrin clot

Figure 142 shows the activation of dilutions of profibrinolysin by constant concentrations of staphylokinase (2 per cent) The yield of fibrinolysin is directly proportional to the amount of profibrinolysin present These experimental observations I think indicate that staphylokinase does act as an enzyme The rate of activation

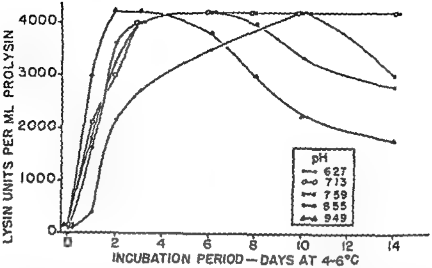

FIGURE 140 Activation of profibrinolysin by staphylokinase Effects of varying pH Reprinted by permission from Lewis J H and Ferguson J H A proteolytic enzyme system of the blood III Activation of dog serum profibrinolysin in staphylokinase *Am J Physiol* 166 594 (1951)

the same fibrinolysin yield was reached but the rate of evolution of fibrinolysin was dependent upon the pH At higher pH levels activation was more rapid but deterioration of formed fibrinolysin was also more rapid At lower pH activation is very slow We chose a pH of 7 for subsequent experiments

In the experiment of Figure 141 we varied the concentration of staphylokinase keeping the concentration of profibrinolysin constant With the exception of the very lowest concentration of staphylokinase the same fibrinolysin yield was obtained from all The control does not activate spontaneously

Seegers In Figure 139 I am interested in the curves for the 0 125 and 0 25 concentrations These curves might be construed as indicating that the activator was actually used in the reaction

Lewis I don t know whether it is Many of our experiments go like this This one we had to stop after four weeks

Seegers Have you explored the possibility of using lower concentrations than 0 125 per cent The reason I ask is because I have found almost without exception that when two things interact the results can be adjusted in such a way as to suggest that one of the reactants has been utilized in the reaction Some individuals have even used such findings to prove that the reaction is stoichiometric I am inclined to the view that manipulations of this type prove nothing of the sort

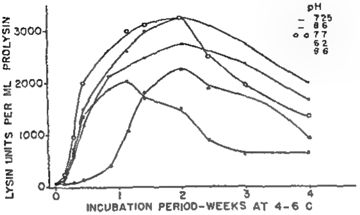

FIGURE 143 Activation of profibrinolysin by lung kinase Effects of varying pH Reprinted by permission from Lewis J H and Ferguson J H The fibrinolytic enzyme system of dog serum *North Carolina M J* 13 196 (1952)

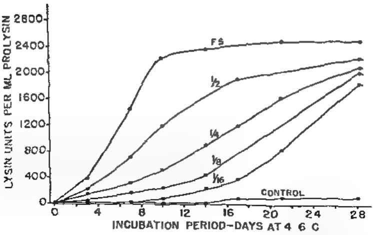

FIGURE 144 Activation of profibrinolysin by lung kinase Effects of varying kinase concentration Reprinted by permission from Lewis J H and Ferguson J H The fibrinolytic enzyme system of dog serum *North Carolina M J* 13 196 (1952)

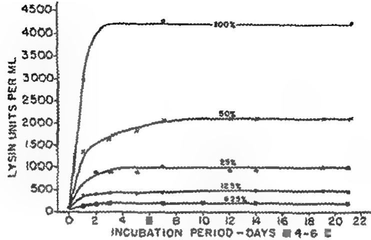

FIGURE 142 Activation of profibrinolysin by staphylokinase Effects of varying profibrinolysin concentration Reprinted by permission from Lewis J H and Ferguson, J H A proteolytic enzyme system of the blood III Activation of dog serum profibrinolysin in staphylokinase *Am J Physiol* 166 594 (1951)

of profibrinolysin is dependent upon the staphylokinase concentration The yield of fibrinolysin is dependent upon the profibrinolysin and independent of the staphylokinase concentration (at least over a certain range)

We next turned to a study of tissue kinase In these experiments we employed the partially purified lung protein Figure 143 shows the effects of pH on the activation of prolysin by this lung kinase A more definite pH optimum is obtained than with staphylokinase We used pH 7 3 in subsequent experiments

In the experiment illustrated in Figure 144 we varied the concentration of lung kinase keeping the concentration of profibrinolysin constant The yield of fibrinolysin from this profibrinolysin treated with staphylokinase was 4200 units per ml As can be seen none of the concentrations of lung kinase gave us the maximal fibrinolysin yield

In the experiment of Figure 145 we diluted the profibrinolysin keeping the lung kinase concentration the same for each test In each case the fibrinoloysin yield was approximately 60 per cent of the potential yield as estimated by separate activation with staphylokinase At the end of the activation period the lung kinase profibrinolysin mixtures would not yield any additional fibrinolysin on treatment with staphylokinase indicating that the profibrinolysin

had been used up A possible explanation for these observations is that under our experimental conditions lung kinase converts pro fibrinolysin to active fibrinolysin and an inert protein

The spontaneous activation of certain fractions and lack of activation in others suggested to me that serum might also contain a fibrinolysokinase substance (54)

Two fractions were prepared from the same lot of serum Both of these fractions euglobulin and prolysin were free from anti fibrinolysin and both yielded approximately 4500 units of fibrinolysin on activation with staphylokinase As shown in Figure 146 the euglobulin activates spontaneously to give a high yield of fibrinolysin The prolysin fraction does not

Alexander This is in the dog?

Lewis In the dog

Alexander No observations in man?

Lewis We have made some preliminary observations in man and we believe the same things hold true

Seegers How did you ever decide to wait so long for your answer? It seems to me that there would have been a temptation to say you didn t get anything the first couple of hours and then quit I think it is an amazing thing to have done

Lewis It takes two or three weeks

Seegers How come you didn t make the mistake of saying it didn t work?

Lewis We knew that at higher temperatures activation occurs somewhat more rapidly but that the formed fibrinolysin deteriorates and we wished to prevent this deterioration and be able to measure the total fibrinolysin yield

Tocantins At room temperature it goes much faster?

Lewis Much faster but deterioration of formed fibrinolysin is also much faster and there is not as great a yield

Jensen We found that trypsin and plasmin (bovine) will activate spontaneously bovine and human profibrinolysin The reaction can be carried out at zero degrees and at room temperature

Lewis I will agree with the trypsin not with the fibrinolysin It is always possible that the fibrinolysin preparation is contaminated with serum fibrinolysokinase which caused it to activate in the first place

Jensen We found that the degree of activation depends a great deal on the amount of the plasmin (fibrinolysin) inhibitor present With the human profibrinolysin preparation kindly supplied by

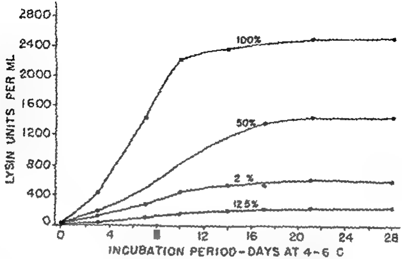

FIGURE 145 Activation of profibrinolysin by lung kinase Effects of varying profibrinolysin concentration Reprinted by permission from Lewis J H and Ferguson J H The fibrinolytic enzyme system of dog serum *North Carolina M J* 13 196 (1952)

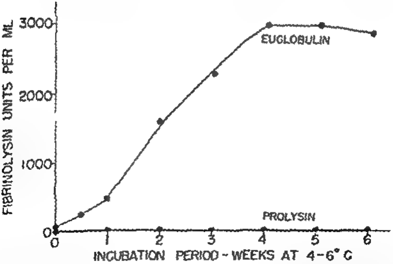

FIGURE 146 Spontaneous activation of prolysin and euglobulin dog serum fractions Reprinted by permission from Lewis J H and Ferguson J H Studies on a proteolytic enzyme system of the blood IV Activation of profibrinolysin by serum fibrinolysokinase *Proc Soc Exper Biol & Med* 78 184 (1951)

minutes Each plasma was divided into two lots one was clotted with thrombin and one with calcium We then treated each with chloroform and observed the spontaneous activation We do not believe that there is any significant difference in the spontaneous activation rates of these four types of serum

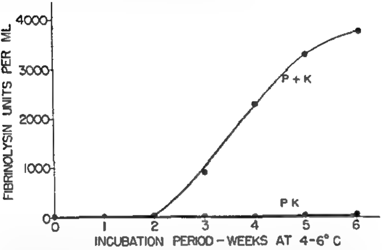

FIGURE 148 Activation of profibrinolysin (P) by serum fibrinolysokinase (K) Reprinted by permission from Lewis J H and Ferguson J H Studies on a proteolytic enzyme system of the blood IV Activation of profibrinolysin by serum fibrinolysokinase *Proc Soc Exper Biol & Med* **78** *184 (1951)*

After a great many trials we isolated from serum a material which will activate our prolysin fraction This material K in Figure 148 contains no profibrinolysin but it does contain a trace of antifibrinolysin If prolysin (P) and serum kinase (K) are incubated separately there is no development of fibrinolysin On the other hand if they are incubated together (P + K) a large yield of fibrinolysin is obtained The lag period in development of fibrinolysin is not adequately explained but we believe that it might be due to the small amount of antifibrinolysin present as a contaminant in our serum kinase fraction

Wright I regret that we shall have to declare this meeting adjourned Thank you for your wonderful cooperation and for tolerating a chairman who has had to be severe at times but knew of no other way to get even this far in attacking our limitless problems

Dr D Surgenor of the Harvard Medical School we obtained very good activation The degree of activation obtained with crystalline trypsin is less than that obtained with streptokinase We may get only 80 per cent activation with trypsin We think that the difference may be due to destruction of activity by trypsin

Lewis Treatment of the prolysin fractions with chloroform does *not cause any activation Treatment with active fibrinolysin* occasionally yields very slow and slight activation which may be due to the serum kinase present in this fibrinolysin. Treatment with trypsin apparently causes complete activation of profibrinolysin therefore we would have to put trypsin into the classification of kinase substances We are in the midst of studying that now

We theorized that there must be a serum kinase substance in the euglobulin fraction (Figure 146) which makes it activate while the prolysin does not It seemed possible that this might be derived

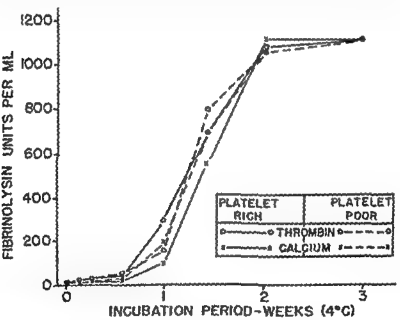

FIGURE 147 The spontaneous activation of fibrinolysin in platelet rich and platelet poor dog sera Reprinted by permission from Lewis J H and Ferguson J H Studies on a proteolytic enzyme system of the blood IV Activation of profibrinolysin by serum fibrinolysokinase *Proc Soc Exper Biol & Med* 78 184 (1951)

from red cells white cells or platelets In the experiment of Figure 147), we prepared platelet rich plasma and platelet poor plasma The platelet poor plasma was centrifuged at 18 000 r p m for 90

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APPENDIX SYNONYMY OF THE NONPROTHROMBIN PLASMA FACTORS INVOLVED IN THE CONVERSION OF PROTHROMBIN TO THROMBIN

BENJAMIN ALEXANDER
Chairman of the Subcommittee
JOSEPH E FLYNN
FRANK D MANN
PAUL A OWREN
WALTER H SEEGERS

AT THE REQUEST of Dr Irving Wright Chairman this subcommittee was appointed to explore the possibility of arriving at some agreement regarding the identity or nonidentity of the various factors reported by numerous investigators which are involved in the physiological conversion of prothrombin to thrombin Factors are listed in alphabetical order by the names of the principal authors The references cited are those which are believed to apply specifically to the various factors but the committee does not intend these citations to indicate the dates at which the investigators formulated their concepts in definitive form since frequently the concepts underwent development and modifications

a) On the basis of available biochemical physiological pathological and clinical information the subcommittee readily agreed that the following factors are one single entity

Prothrombin Accelerator of Fantl and Nance (1 2)
Plasmatic Cofactor of Thromboplastin of Honorato (3 4)
Proaccelerin (Factor V) of Owren (5 6 7 8)
Labile Factor (Prothrombin A of 1943) of Quick (9 10)
Plasma Prothrombin Conversion Factor (PPCF) of Stefanini (11)
Plasma Accelerator (Ac) Globulin of Ware Guest and Seegers (12 13)

This probably is the substance suspected and referred to much earlier by the Iowa group as the "convertibility factor (14 15 16 17)

The committee decided it was not their prerogative to determine which was the most appropriate term to designate this substance but a few believed that proaccelerin or plasma Ac globulin were well suited for the purpose of simplifying nomenclature

In connection with this factor the subcommittee discussed the substance in platelets which exhibits Ac globulin like activity (18)

Except for Drs Flynn and Mann (vide infra) the members also

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agreed that during the course of spontaneous coagulation of mammalian blood or under the influence of added thrombin proaccelerin (plasma Ac globulin) which is relatively inert is converted to accelerin (serum Ac globulin) the more active form

b) The subcommittee agreed furthermore that mammalian plasma contains another factor important in the physiologic conversion of prothrombin to thrombin This substance distinct from that described above has been referred to by the following terms

Spca precursor of Alexander *et al* (19 20 21 22 23)

Factor VII of Koller *et al* (24 25)

Co Thromboplastin of Mann and his colleagues (26 27 28 29)

Stable Prothrombin Conversion Factor of Owen et al (30 31 32)

Proconvertin (co Factor V) of Owren (7 8 33 34 35 36)

With the exception of Dr Mann the subcommittee members concurred that during the course of coagulation this substance was converted to a more active entity termed spca or convertin Dr Mann was not convinced that the tests employed were sufficiently specific to warrant the conclusion that the conversion factors described under (*a*) and (*b*) are initially inactive and must be converted to active forms before taking part in coagulation although he did not deny the possibility

c) The subcommittee discussed other entities and clotting activities described by numerous investigators Due cognizance was taken of Bordet s early observations of greater clot promoting activity of serum as contrasted with plasma (37 38) of Nolf's concepts (39) of plasmakinin of Laki (40) of the prothrombokinase thrombokinase system and globulin substance of Milstone (41 42) of the prothrombin converting factor of Jacox (43) and of the kappa and delta factors of Sørbye *et al* in chicken plasma (44 45) It was generally believed that our present state of knowledge does not permit definitive conclusions as to their relationship with one another or with the substances described above

The foregoing does not encompass consideration of the nature of thromboplastin the plasma entity which together with platelets is necessary for the elaboration of thromboplastic material in coagulating blood or factors which inhibit the interaction of the above mentioned components

No consideration was given to the matter of priority in the discovery of any of these factors Omission of any entity or of any investigator who has reported a factor involved in prothrombin conversion is unintentional and stems from incomplete acquaintance with the literature

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CUMULATIVE INDEX FOR THE TRANSACTIONS OF THE CONFERENCES 1948 1952

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Volume		
1949	p 173 line 39	for J Clint Invest read "J Clin Investigation"
1949	p 219 line 20	for "interrelations read interpretations
1950	Table of Contents p 6 item 5	for L M Tocantis read "L M Tocantins
1950	p 11 title	for Eric Lepp" read Erica Lepp
1950	p 22 line 16	insert next before dose
1950	p 28 line 23	for affect" read "effect"
1950	p 68 footnote 2	for Eisensauer read Eisenhauer
1950	p 74 line 17	for radioactivity read "activity
1950	p 78 legend Fig 18	interchange explanation of symbols lines 2 and 3
1950	p 81 line 25	for radioctivity" read "radioactivity"
1950	p 104 caption Table XIV	for anticoaguants read anticoagulants
1950	p 216 title	for John A Ferguson read John H Ferguson
1950	p 217 line 4	insert comma after "fibrinogen"
1950	p 218 caption Table XXX	for plasama read "plasma
1950	p 218 line 10	for 1 2" read "1 2